DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service
Centers for Disease Control and Prevention (CDC)
Atlanta, GA 30329

Manual Procedure Illumina DNA Library Preparation (Quarter Volume Reaction)

Virology, Surveillance and Diagnosis Branch, Genomics and Diagnostics Team (GDT)

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1.0 Purpose

1.1 This protocol describes the manual procedure for preparing libraries from purified MRT-PCR Influenza A and B amplicons, using quarter reaction volumes from the Illumina DNA Library Sample Preparation Kit and IDT for Illumina DNA/RNA UD Index Kits.

2.0 Definitions

Term	Definition
EtOH	Ethanol
IPB	Illumina Purification Beads
PPE	Personal Protective Equipment
BLT	Bead Linked Transposome
EPM	Enhanced PCR Mix
RSB	Resuspension Buffer
TB1	Tagmentation Buffer 1
TSB	Tagmentation Stop Buffer
TWB	Tagmentation Wash Buffer
UD	Unique Dual

3.0 Critical Equipment

3.1 96-well thermal cycler with heated lid
3.2 Vortex
3.3 Minicentrifuge with 1.5 ml and 0.5 ml strip tube rotors
3.4 Tabletop centrifuge for 96-well plates

4.0 Laboratory Materials

4.1 Pipettes (2 µl, 10 µl, 20 µl, 200 µl, and 1000 µl)

4.2 Aerosol barrier pipette tips

4.3 PCR Plate, 96-well (hard-shell, full skirt)

4.4 PCR Plate Sealing Film (USA Scientific: Catalog No. 2923-0110 or equivalent)

4.5 PCR 8-tube strips, caps clear (Brand Tech Scientific Inc. Catalog. No. 781332)

4.6 DynaMag 96 Side Magnet (Invitrogen Cat# 1038378-0 or equivalent)

4.7 25 ml disposable reagent reservoir, sterile (Axygen: Catalog No. RES-V-25-S or equivalent)

4.8 Sealing Roller (BIO-RAD: Catalog No. MSR-0001 or equivalent)

4.9 Ultra-Pure Water (Molecular Biology Grade) Nuclease Free

4.10 DNA Template (MRT-PCR Influenza A and/or B amplicons)

4.11 Ethanol, 200 proof, molecular biology grade

4.12 Illumina DNA LP (M) Tagmentation (IPB) (Illumina Cat# 20060059).

4.12.1 Illumina DNA Prep – Beads and Buffers

Reagent Name	Acronym	Storage
Illumina Purification Beads	IPB	Stored at 15°C to 25°C.
Tagmentation Stop Buffer	TSB	Stored at 15°C to 25°C.
Tagmentation Wash Buffer	TWB	Stored at 15°C to 25°C.

4.12.2 Illumina DNA Prep – PCR and Buffers

Reagent Name	Acronym	Storage
Resuspension Buffer	RSB	Stored at -25°C to -15°C.
Tagmentation Buffer 1	TB1	Stored at -25°C to -15°C.
Enhancement PCR Mix	EPM	Stored at -25°C to -15°C.

4.12.3 Illumina DNA Prep – Tagmentation (M) beads

Reagent Name	Acronym	Storage
Bead-Linked Transposomes	BLT	Stored at 2°C to 8°C.

4.13 **IDT for Illumina DNA/RNA UD Indexes, Tagmentation:**

Description (Set)	Product #	Storage
Set A	20027213	Stored at -25°C to -15°C.
Set B	20027214	Stored at -25°C to -15°C.
Set C	20042666	Stored at -25°C to -15°C.
Set D	20042667	Stored at -25°C to -15°C.

5.0 Controls

5.1 A library negative template control containing water (LIB NTC) is added to all processing plates.

6.0 Safety Precautions

6.1 Adhere to the safety guidelines provided in the Biosafety in Microbiological and Biomedical Laboratories and follow all established site-specific safety procedures, including wearing proper personal protective equipment (PPE).

7.0 Procedure

7.1 The Illumina DNA Library Preparation Kit, using quarter reaction volumes, allows for the processing of four 96-well plates (384 reactions).

7.2 The IDT for Illumina DNA/RNA UD Index Kit, using quarter reaction volumes, allows for the processing of four 96-well plates (384 reactions).

7.3 **Tagment Genomic DNA**

7.3.1 Prepare Tagmentation Bead solution:

- Bring BLT and TB1 to room temperature. Vortex to mix.
- Prepare enough to for all samples and controls and account for 10% overage.

Table 1: Tagmentation Master Mix		
Reagent	Volume (µl) Per Reaction	µl Per Master Mix
BLT	2.5	
TB1	2.5	

7.3.2 Divide the master mix into an 8-well strip tube for easy transferring.

7.3.3 For each sample, transfer 5 µL of the Tagmentation master mix to the wells of a new 96-well hard-shell PCR plate.

7.3.4 Add 7.5 µL of 1:24 diluted MRT-PCR amplicons or 7.5 µl of nuclease free water for LIB NTC to each well containing master mix. Pipette 10 times to resuspend.

7.3.5 Seal the plate, place on thermal cycler and run the TAG program.

Table 2: Tagmentation (TAG) incubation conditions	
Temperature (°C)	**Time (mm:ss)**
55	15:00
10	∞

7.4 **Post Tagmentation Cleanup**

7.4.1 Vortex to mix TSB.

7.4.2 Add 2.5 µl of TSB to each sample in the plate.

7.4.3 Slowly pipette each well 10 times to resuspend the beads, and then seal plate.

7.4.4 Place on the preprogrammed thermal cycler and run the PTC program.

Table 3: Post-Tagmentation Cleanup (PTC) incubation conditions	
Temperature (°C)	**Time (mm:ss)**
37	15:00
10	∞

7.4.5 Move plate to a magnetic stand until liquid is clear (~3 min).

7.4.6 Remove supernatant and discard.

7.4.7 Remove plate from magnet and **slowly** add 25 µl of TWB. Pipette slowly to minimize the potential of foaming.

7.4.8 Pipette **slowly** to resuspend or use shaker at 1600 rpm for 1 minute. Ensure beads are fully resuspended.

7.4.9 Repeat steps 9.4.5 to 9.4.8 twice for a total of three washes.

7.4.10 Keep beads and TWB (from third wash) bound to plate on magnet and proceed directly to next step.

7.5 **Amplify Tagmented DNA**

7.5.1 Thaw EPM on ice.

7.5.2 Thaw index adapters at room temperature and centrifuge plate briefly before use.

7.5.3 Dilute EPM as per master mix below. Volumes already contain 10% excess.

Table 4: Enhancement PCR Master Mix		
Reagent	Volume (µl) Per Reaction	µl Per Master Mix
EPM	5.5	
Nuclease-free water	5.5	

7.5.4 Remove the supernatant from plate (step 9.4.10) and discard.
- Foam remaining on well will not negatively affect library produced.

7.5.5 Remove plate from magnet and add 10 µl of the diluted Enhancement PCR master mix.

7.5.6 Gently pipette to mix or seal and shake plate at 1600 rpm for 1 minute. Ensure beads are fully resuspended.

7.5.7 Seal plate and centrifuge plate at 280 x g for 3 seconds.

7.5.8 Add 2.5 µl of IDT for Illumina DNA/RNA UD index adapters to each sample.

7.5.9 Each index adapter well contains a pre-paired i7 and i5 index adapter. Gently pipette to mix or seal and shake plate at 1600 rpm for 1 minute.

7.5.10 Seal plate and then centrifuge at 280 × g for 30 seconds.

7.5.11 Place on the preprogrammed thermal cycler and run BLT PCR program.
- Choose preheat lid option and set to 100°C.

Table 5: Bead Linked Transposome (BLT) PCR cycling conditions		
Temperature (°C)	**Time (mm:ss)**	**# Cycles**
68	3:00	1
98	3:00	1
98	0:45	7
62	0:30	
68	2:00	
68	1:00	1
10	∞	1

7.5.12 **SAFE STOPPING POINT**
- Libraries can be stored at 2°C to 8°C for up to 30 days.

7.6 **Clean Up Libraries**

7.6.1 Thaw RSB and bring to room temperature. Ensure that IPB is at room temperature.

7.6.2 Prepare fresh 80% ethanol (minimum of 100 µl per sample).

7.6.3 Centrifuge BLT PCR plate at 280 × g for 1 minute.

7.6.4 Move plate to a magnetic stand and wait until liquid is clear (~5 min).

7.6.5 Transfer 11 µl supernatant from each well of the 96-well PCR plate to the corresponding well of a new 96-well hard-shell PCR plate.

7.6.6 Vortex IPB to resuspend and add 20 µl of IPB to each sample in the new PCR plate.

7.6.7 Gently pipette to mix or seal and shake plate at 1600 rpm for 1 minute.

7.6.8 Incubate at room temperature for 5 min.

7.6.9 Move plate to a magnetic stand until liquid is clear.

7.6.10 Remove the supernatant from plate and discard.

7.6.11 With plate on magnet, add 50µl of fresh 80% ethanol without disturbing beads.

- Incubate for 30 seconds.

7.6.12 Remove and discard ethanol.

7.6.13 Repeat steps 9.6.11-9.6.12 for a total of two washes.

- Plate can be sealed, briefly centrifuge, and returned to magnet to remove excess ethanol.

7.6.14 Air-dry for 5 minutes or until beads appear dry.

- Do not over dry pellets.

7.6.15 Remove plate from magnetic stand.

7.6.16 Add 15 µl of RSB.

7.6.17 Pipette gently to resuspend.

7.6.18 Incubate at room temperature for 2 minutes.

7.6.19 Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

7.6.20 Transfer 12 µl supernatant to a new PCR plate.

7.6.21 SAFE STOPPING POINT.

- Libraries can be stored at 2°C to 8°C for up to 30 days.

7.7 **Pool Libraries**

7.7.1 Using a multichannel pipette, transfer 5 µl of each library into a strip tube.

7.7.2 After transferring all samples, combine all into one 1.5 ml tube.

7.7.3 SAFE STOPPING POINT

- Pooled libraries can be stored at 2°C to 8°C.

8.0 Related Procedures

8.1 LP-328 - Multi-segment Reverse Transcription-PCR (M-RTPCR) of Influenza A and B Viruses

8.2 LP-347 - MRT-PCR Purification using Exonuclease I

8.3 LP-325 - QIAxcel Sample Preparation and QC Procedure

8.4 LP-516 - DNA Quantification with Qubit dsDNA High Sensitivity Kit

9.0 References

9.1 Illumina DNA Prep - Reference Guide, Document # 1000000025416 v10, August 2021 https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/illumina_prep/illumina-dna-prep-reference-guide-1000000025416-10.pdf

10.0 Attachments

10.1 N/A