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Illumina Microbial Amplicon Prep—Influenza A/B

Reference Guide

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Table of Contents

Overview

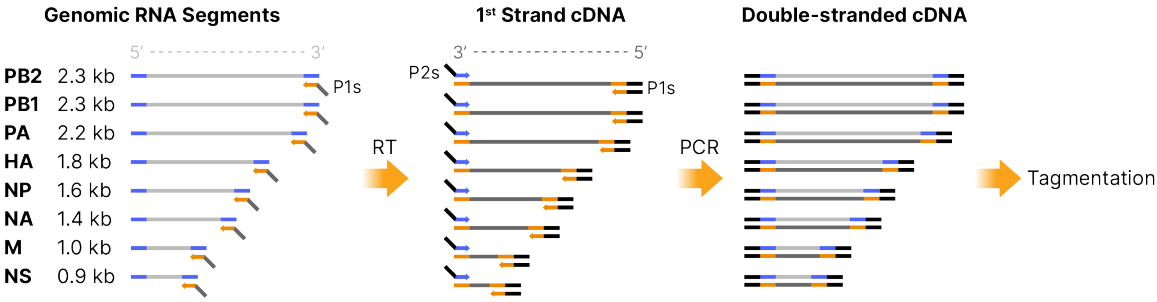
This resource explains how to prepare up to 48 uniquely indexed influenza A or influenza B targeted genomic libraries from total RNA inputs using the Illumina Microbial Amplicon Prep—Influenza A/B (IMAP-Flu) workflow. This kit enables and includes the required primer sets for whole genome sequencing of influenza A/B viruses. The result is a targeted whole genome influenza A/B virus library from RNA for sequencing on Illumina systems. Results may vary depending on the concentration of virus in the sample and are not guaranteed.

IMAP-Flu offers:

- Preparation of ≤ 48 unique dual-indexed libraries
- Scalable library prep from 1–48 samples
- Generation of sequence-ready libraries in < 9 hours
- Assay of Influenza A and Influenza B subtypes

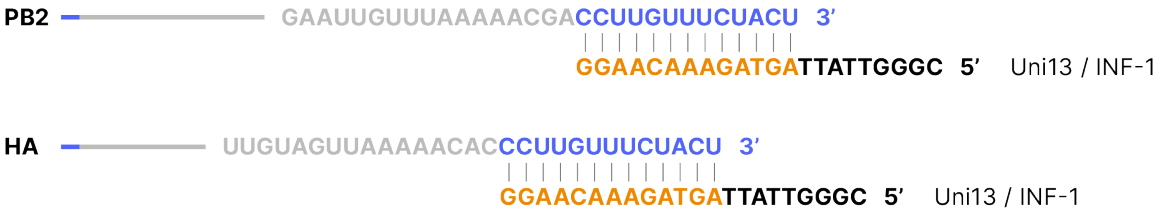
Universal influenza virus whole genome RT-PCR

Figure 1



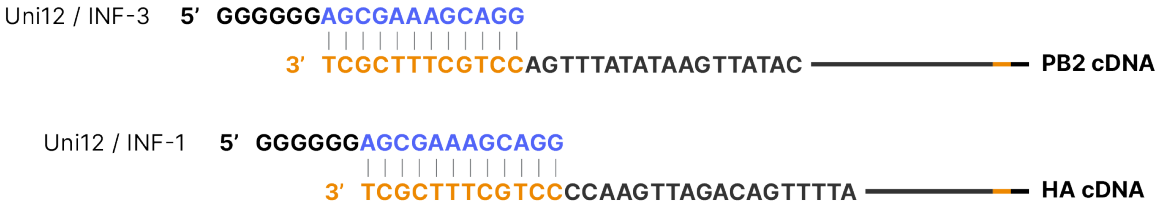
Influenza whole genome RT-PCR approach to generate amplicons ready for tagmentation. The Influenza A/B Primer Pool contains 16 primers designed to hybridize to either the 3′ ends of the genomic RNA segments and 2nd strand cDNA or the 3′ ends of the 1st strand cDNA, enabling both cDNA synthesis of each genomic RNA segment and subsequent PCR amplification to produce whole genome amplicons ready for tagmentation. Blue lines represent the conserved non-coding regions of each genomic RNA segment (or 2nd strand cDNA), the orange lines the complementary portions of the P1 primers, and the black lines represent the additional DNA sequences added by the universal primers to increase primer melting temperatures during PCR.

P1 primer alignment examples to genomic RNA



Primer binding site examples for P1 primers to 3′ end of genomic RNAs using the influenza A virus (A/swan/Germany/R65/2006(H5N1)) genomic sequences as target examples. Blue sequences represent complementary sequences of the 3′ ends of the genomic RNAs (or 3′ ends of second strand cDNA) where P1 primers bind.

P2 primer alignment examples to 1st strand cDNA



Primer binding site examples for P2 primers binding to the 3′ end of the 1st strand cDNA using influenza A virus (A/swan/Germany/R65/2006(H5N1)) genomic sequences converted to cDNA as target examples.

Input Recommendations

The following section provides input recommendations.

Assess RNA Sample Purity

IMAP-Flu supports RNA inputs from various sample types. To improve library preparation success, use the highest quality nucleic acid input. It is good practice to assess sample quality before beginning targeted library prep.

UV absorbance is a common method used for assessing the purity of a sample. The ratio of absorbance at 260 nm to absorbance at 280 nm provides an indication of sample purity from protein. This protocol is optimized for RNA with 260/280 absorbance ratio values of 2.0–2.2 which indicates a high purity sample.

For a secondary indication of sample purity, use the ratio of absorbance at 260 nm to absorbance at 230 nm. Target a 260/230 ratio of 2.0–2.2. Values outside this range indicate the presence of organic chemical contaminants such as ethanol, guanidine salts, or phenol, which can carryover from the nucleic acid extraction method. Using nucleic acids that do not meet these recommendations may result in assay failure. If you experience assay failure, Illumina recommends cleaning up the samples and repeating library preparation.

Purity Check	RNA
A260/280	2.0–2.2
A260/230	2.0–2.2

Input Amount

Given the highly variable target abundance in sample types used with this technology, Illumina recommends using the CDC qRT-PCR protocol[1] or the Ward protocol[2] for measuring influenza A and influenza B viral loads, and samples with Ct values < 30 for high sequencing depth and complete whole genome coverage.

Purification Kits

Many commercially available RNA extraction and purification kits are available. Choose a kit or method that produces the highest quality nucleic acids possible for your specific targets and from your specific sample types. Illumina tested the following kits. Other kits or methods may be preferred for your samples and targets.

- Quick-DNA/RNA Viral MagBead, Zymo Research, part # R2141.
- ZymoBIOMICS DNA/RNA Miniprep Kit, Zymo Research, part # R2002.
- QIAamp Viral RNA Mini Kit, QIAGEN, part # 52906 and 52904.

1. Bo S, Kai-Hui W, Shannon E, et al. Design and Performance of the CDC Real-Time Reverse Transcriptase PCR Swine Flu Panel for Detection of 2009 A (H1N1) Pandemic Influenza Virus. Journal of Clinical Microbiology. Published July 2011. Accessed September 15, 2023. https://journals.asm.org/doi/10.1128/jcm.02636-10.

2. Ward CL, Dempsey MH, Ring CJA, et al. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. Journal of Clinical Virology. Published March 2004. Accessed September 15, 2023. https://www.sciencedirect.com/science/article/pii/S1386653203001227.

Consumables and Equipment

The IMAP-Flu protocol requires the following Illumina-supplied and user-supplied consumables and equipment.

The protocols have been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Make sure that you have the required consumables and equipment before starting the protocol.

Product Contents

Each library prep reagent kit comprises five boxes. The following tables list the contents of each box. Promptly store reagents at the indicated temperature to ensure proper performance.

Illumina Microbial Amplicon Prep Box 1, Store at Room Temperature

Table 1 Box 1 — 48 Samples, Component Box # 20093449

Quantity	Reagent	Description
1	IPB	Illumina Purification Beads
1	ST2	Stop Tagment Buffer 2

Illumina Microbial Amplicon Prep Box 2, Store at 2°C to 8°C

Table 2 Box 2 — 48 Samples, Component Box # 20093450

Quantity	Reagent	Description
1	ELB	Elution Buffer
1	EBLTS	Enrichment BLT
1	TWB	Tagmentation Wash Buffer

Illumina Microbial Amplicon Prep Box 3, Store at -25°C to -15°C

Table 3 Box 3 — 48 Samples, Component Box # 20093451

Quantity	Reagent	Description
2	EPH3	Elution Prime Fragment 3HC Mix
2	EPM	Enhanced PCR Mix
2	FSM	First Strand Mix
2	IPM	Illumina PCR Mix

Quantity	Reagent	Description
2	RSB	Resuspension Buffer
1	RVT	Reverse Transcriptase
3	TB1	Tagmentation Buffer 1

Illumina Unique Dual Indexes, LT, Store at -25°C to -15°C

Table 4 Illumina Unique Dual Indexes, LT — 48 Samples, Box # 20083060

Quantity	Description
1	Illumina Unique Dual Indexes, LT

Illumina Microbial Amplicon Prep—Influenza A/B Primer Pool, Store at -25°C to -15°C

Table 5 Influenza A/B Primer Pool— 48 Samples, Box # 20108095

Quantity	Reagent	Description
1	IPP	Influenza A/B Primer Pool

User-Supplied Consumables and Equipment

Completing the IMAP-Flu protocol requires the following user-supplied consumables and equipment.

Consumables

Consumables	Supplier
Tubes, 15 ml	General lab supplier
Pipette tips, 10 µl	General lab supplier
Pipette tips, 20 µl	General lab supplier
Pipette tips, 200 µl	General lab supplier
Pipette tips, 1000 µl	General lab supplier
Hard-shell 96-well PCR plates with the following specifications: • 96 wells with 0.2 ml well capacity • Polypropylene or equivalent • Compatible with the thermal cycler used • Full or semi-skirted	General lab supplier
8-tube strips	General lab supplier
1.7 ml LoBind microcentrifuge tubes	Eppendorf, catalog # 022431021

Consumables	Supplier
Lint-free alcohol wipe	General lab supplier
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
RNase/DNase-free Disposable Pipetting Reservoirs	VWR, part # 89094-658
Qubit dsDNA HS Assay Kit	One of the following, depending on kit size: • Thermo Fisher Scientific, part # Q32851 • Thermo Fisher Scientific, part # Q32854
Qubit Assay Tubes	Thermo Fisher Scientific, catalog # Q32856
Ethanol (EtOH) 100%, molecular biology grade	General lab supplier

Equipment

Equipment	Supplier
Pipettes, single-channel, 10 µl	General lab supplier
Pipettes, single-channel, 20 µl	General lab supplier
Pipettes, single-channel, 200 µl	General lab supplier
Pipettes, single-channel, 1000 µl	General lab supplier
Pipettes, 8-channel, 10 µl	General lab supplier
Pipettes, 8-channel, 20 µl	General lab supplier
Pipettes, 8-channel, 200 µl	General lab supplier
Pipettes, 8-channel, 1000 µl	General lab supplier
Pipettes, 12-channel, 20 µl	General lab supplier
Pipettes, 12-channel, 200 µl	General lab supplier
BioShake iQ	QINSTRUMENTS, part # 1808-0506
DynaMag-2 Magnet	Thermo Fisher, catalog # 12321D
Magnetic Stand-96	Thermo Fisher, catalog # AM10027
Microcentrifuge	General lab supplier
Microplate centrifuge	General lab supplier

Equipment	Supplier
Qubit Fluorometer • 3.0 • 4.0 (instrument) • 4.0 (instrument and starter kits)	Thermo Fisher • catalog # Q33216, Q33217, or Q33218 • catalog # Q33238 • catalog # Q33239, Q33240, Q33241, or A51292
Refrigerator, 2°C to 8°C	General lab supplier
Freezer, -15°C to -25°C	General lab supplier
Sealing wedge or roller	General lab supplier
Vortexer	General lab supplier

Thermal Cycler

The following table lists recommended thermal cyclers or thermal cycler specifications. PCR thermal cyclers must be capable of supporting the sample volumes and temperature profiles used in this workflow, with appropriate thermal accuracy and block uniformity to ensure consistent incubation and amplification performance. Validate the thermal cycler before performing the protocol.

Performance may vary depending on the specific thermal cycler and consumables used. Minor workflow optimization may be required to account for instrument and consumable specific differences.

Thermal Cycler	Supplier

Thermal cycler with the following specifications:
• Heated lid, max temp ≥ 105°C
• Block ramp rate: ≥ 2.5°C/sec
• Temperature control range:
  • Min ≤ 4°C
  • Max ≥ 99°C
• Temperature accuracy: ±0.25°C
• Temperature uniformity: ±0.5°C
• Capable of supporting reaction volumes of 100 µl
• Compatible with 96-well PCR plates (full or semi-skirted), or suitable for the applicable workflow.
	General lab supplier

Protocol

This section describes library preparation using the IMAP-Flu kit.

The following protocol has been tested with multiple subtypes of influenza A/B viruses at different viral loads.

This protocol is designed for library preparation in 96-well PCR plates. If the sample number is low and better suited to library preparation in 1.5 ml microcentrifuge tubes or strip tubes, modify the protocol as necessary.

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Recommendations

- Make sure that reagents are not expired. Using expired reagents may negatively impact performance.
- Mix reagents thoroughly prior to use.
- Do not allow more than eight freeze-thaw cycles for any reagent in this kit.
- Running a negative control is recommended to alert you to potential amplicon carryover from previous experiments. Elution Buffer (ELB) is provided to run as a no template control. There should be no amplicons produced from the negative control samples.
- Running a positive control is recommended, but may not be required. You can use RNA diluted with ELB as a positive control.
- Sequence libraries as soon as possible after pooling. Pooled libraries are stable for up to 30 days at -25°C to -15°C.

Avoiding Contamination

- Use proper laboratory practices to prevent nuclease and PCR product contamination. Nuclease and PCR product contamination can cause inaccurate and unreliable results.
- Perform library preparation in a RNase/DNase-free environment. Thoroughly decontaminate work areas with a RNAse/DNase-inhibiting solution, such as RNAseZap and DNAZap.
- Use fresh tips and fresh consumable labware between samples and dispensing reagents.
- Use aerosol-resistant tips to reduce the risk of carry-over and sample-to-sample cross-contamination.
- Due to the potential for contamination, take extreme care to make sure that well contents remain fully in the well. Do not splash contents.
- Do not use aerosol bleach sprays when performing library preparation. Trace bleach contamination can lead to assay failure.

- Use a unidirectional workflow when moving from pre-amplification to post-amplification environments.
- One or more no template controls (NTCs) are recommended per plate to monitor contamination.

Sealing and Unsealing the Plate

- This protocol frequently instructs users to "seal" 96-well plates; this step is critical before executing the following workflow steps:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps
  - Overnight or long-term storage
- In all cases, seal plates using Microseal 'B' adhesive. Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semi-skirted PCR plates.
- Use Microseal 'B' for shaking, centrifuging, and long-term storage.
- When applying Microseal 'B' to seal a 96-well plate, use a wedge or rubber roller against all sample wells and plate edges to ensure thorough adhesion. All sample wells must be completely sealed to prevent cross-contamination or evaporation.
- Before unsealing:
  - Briefly centrifuge the 96-well plate at 1000 × g for 1 minute. For bead steps, centrifuge at 500 × g for 1 minute.
  - Place the plate on a flat surface before slowly removing the seal.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.
- If beads are aspirated into the pipette tips, dispense back to the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

Centrifugation

- Centrifuge as needed at any step in the procedure to consolidate liquid or beads in the bottom of the well, and to prevent sample loss.

Handling Beads

- Pipette bead suspension slowly to prevent splashing and bubbles.
- When mixing, mix thoroughly.
- To avoid sample loss, confirm that no beads remain in pipette tips after resuspension and mixing steps.
- When washing beads:
  - Use the appropriate magnet for the plate.

- Dispense liquid so that beads on the side of the wells are wetted.
- Keep the plate on the magnet until the instructions specify to remove it.
- Do not agitate the plate while on the magnetic stand. Do not disturb the bead pellet.

Prepare for Protocol

1. Remove RNA samples from storage.
2. Remove the reagents and prepare as follows.

Table 6 -25°C to -15°C Storage

Reagent	Box Name	Instructions
FSM	Box 3	Thaw at room temperature.
RVT	Box 3	Thaw on ice.
IPM	Box 3	Thaw at room temperature.
IPP	Illumina Microbial Amplicon Prep—Influenza A/B Primer Pool	Thaw at room temperature.

cDNA Synthesis and Amplicon PCR

This step uses the Influenza A/B Primer Pool (IPP) to amplify 8 genomic RNA segments from each RNA sample to produce the amplicons that will be tagmented.

This portion of the protocol is designed to use one primer pool that targets the whole genome of influenza A and B viruses using universal primers designed to anneal to the ends of each genomic RNA in RNA or cDNA form.

Consumables

- FSM (First Strand Mix)
- RVT (Reverse Transcriptase)
- IPM (Illumina PCR Mix)
- IPP (Influenza A/B Primer Pool)
- Nuclease-free water
- 1.7 ml tube
- 96-well PCR plates (1)
- Microseal 'B' adhesive seal

Preparation

1. Prepare the following consumables:
   - FSM—Invert to mix. Keep on ice until use. If necessary, vortex to mix and eliminate all precipitation.
   - IPM—Invert to mix. Keep on ice until use. If necessary, vortex to mix and eliminate all precipitation.
   - IPP—Invert to mix. Keep on ice until use.
   - RVT—Invert to mix. Keep on ice until use.
2. Save the following IMAP-Flu PCR program on the thermal cycler:
   - Choose the preheat lid option at 105°C
   - Set the reaction volume to 25 µl
   - 42°C for 60 minutes
   - 94°C for 2 minutes
   - 5 cycles of:
     - 94°C for 20 seconds
     - 44°C for 30 seconds
     - 68°C for 3 minutes
   - 35 cycles of:
     - 94°C for 20 seconds
     - 58°C for 30 seconds
     - 68°C for 3 minutes
   - 68°C for 5 minutes
   - Hold at 4°C
3. Label a new PCR plate RT-PCR.

Procedure

1. In a 1.7 ml tube, combine the following reagents to prepare RT-PCR Master Mix. Multiply each volume by the number of samples.
   Reagent overage is included to account for small pipetting errors.

Reagent	RT-PCR Master Mix (µl)
IPM	15
FSM	3.2

Reagent	RT-PCR Master Mix (µl)
IPP	1.2
RVT	1.0
Nuclease-free water	3.6

2. Manually mix by gently pipetting, aspirating, and discharging ~50% total volume 5-10 times.
3. Add 20 µl RT-PCR Master Mix to each well of the RT-PCR plate.
4. Add 5 µl RNA to each well of the RT-PCR plate.
5. Seal and shake at 1600 rpm for 1 minute.
6. Centrifuge at 1000 x g for 1 minute.
7. Place in the preprogrammed thermal cycler and run the IMAP-Flu PCR program.

Clean Up PCR Product

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- EtOH (absolute ethanol)

About Reagent

- IPB (Illumina Purification Beads)
  - Vortex frequently to make sure of even distribution.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

Prepare the following consumables:

- IPB—Vortex to mix.
- RSB—Thaw on ice. Vortex and invert to mix.
- 80% EtOH—Prepare 400 µl 80% EtOH from absolute EtOH for each well of PCR product.

Procedure

1. Vortex and add 15 µl of IPB to each 25 µl RT-PCR reaction.
2. Shake at 2200 rpm for 1 minute.
3. Incubate at room temp for 5 minutes.

4. Centrifuge at 280 × g for 3 seconds. Place on a magnetic stand and wait until the liquid is clear.
5. Remove and discard all supernatant.
6. Wash beads as follows:
   a. Add 175 µl 80% EtOH to each well.
   b. Wait for 30 seconds.
   c. Remove all supernatant and discard.
7. Wash beads a **second** time.
8. Dry the beads at room temperature for 2 min.
9. Add 23 µl RSB to each well, and remove from the magnetic stand.
10. Shake at 2200 rpm for 1 minute.
11. Incubate at room temperature for 2 minutes.
12. Centrifuge at 280 × g for 3 seconds.
13. Place on a magnetic plate stand and wait until liquid is clear.
14. Transfer 20 µl of supernatant from each well to the new PCR plate labeled TAG1.

SAFE STOPPING POINT

If you are stopping, seal the TAG1 plate and store at -25°C to -15°C for up to 3 days.

Prepare for Protocol

1. Remove the TAG1 plate from storage.
2. Remove the reagents and prepare as follows.

Table 7 Room Temperature Storage

Reagent	Box Name	Instructions
IPB	Box 1	Use at room temperature.
ST2	Box 1	Use at room temperature.

Table 8 2°C to 8°C Storage

Reagent	Box Name	Instructions
EBLTS	Box 2	Use at room temperature.
TWB	Box 2	Use at room temperature.

Table 9 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EPM	Box 3	Thaw on ice.

Reagent	Box Name	Instructions
Index adapter	Illumina Unique Dual Indexes, LT	Thaw at room temperature.
RSB	Box 3	Thaw at room temperature.
TB1	Box 3	Thaw at room temperature.

Tagment PCR Amplicons

This step uses EBLTS to tagment the influenza A/B virus whole genome RT-PCR amplicons. The tagmentation process fragments and tags the amplicons with adapter sequences.

Consumables

- EBLTS (Enrichment BLT)
- TB1 (Tagmentation Buffer 1)
- Nuclease-free water
- 1.7 ml tube
- 2 ml tube
- 96-well PCR plate
- Microseal 'B' adhesive seal

About Reagents

- EBLTS
  - Store upright at temperatures above 2°C. Make sure beads are always submerged in the buffer.
  - If beads are adhered to the side or top of the wells, centrifuge at 500 × g for 1 minute, and then pipette to resuspend.

Preparation

1. Prepare the following consumables:
   - EBLTS—Vortex to mix.
   - TB1—Vortex to mix.
2. If the TAG1 plate was stored frozen, prepare as follows:
   a. Thaw at room temperature.
   b. Check seals, and then shake at 1600 rpm for 1 minute.
   c. Centrifuge at 1000 × g for 1 minute.

3. Save the following IMAP-Flu TAG program on the thermal cycler:
    - Choose the preheat lid option at 105°C
    - Set the reaction volume to 50 µl
    - 55°C for 5 minutes
    - Hold at 10°C

Procedure

1. In a 2 ml tube, combine the following volumes to prepare Tagmentation Master Mix. Multiply each volume by the number of samples. Reagent overage is included to account for small pipetting errors.
    - TB1 (12 µl)
    - EBLTS (4 µl)
    - Nuclease-free water (20 µl)
2. Pipette the Tagmentation Master Mix thoroughly to mix.
3. Add 30 µl Tagmentation master mix to each well of the TAG1 plate.
4. Seal and shake at 1600 rpm for 1 minute.
5. Place on the preprogrammed thermal cycler and run the IMAP-Flu TAG program.

Post Tagmentation Clean Up

This step washes the adapter-tagged amplicons before PCR indexing.

Consumables

- ST2 (Stop Tagment Buffer 2)
- TWB (Tagmentation Wash Buffer)
- Microseal 'B' adhesive seal

About Reagents

- ST2 and TWB—Dispense slowly to minimize foaming.
- TWB—Dispense directly onto beads.

Preparation

Prepare the following consumables:

- ST2—Vortex before use.
- TWB—Vortex before use.

Procedure

1. Centrifuge the TAG1 plate at 500 x g for 1 minute.
2. Add 10 µl ST2 to each well of the TAG1 plate.
3. Seal and shake at 1600 rpm for 1 minute.
4. Incubate at room temperature for 5 minutes.
5. Centrifuge at 500 × g for 1 minute.
6. Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
7. Inspect for bubbles on the seal. If bubbles are present, centrifuge at 500 × g for 1 minute, and then place on the magnetic stand (~3 minutes).
8. Remove and discard all supernatant.
9. Wash beads as follows.
    a. Remove from the magnetic stand.
    b. Add 100 µl TWB to each well.
    c. Seal and shake at 1600 rpm for 1 minute.
    d. Centrifuge 500 × g for 1 minute.
    e. Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
    f. For the first wash only, remove and discard all supernatant from each well.
10. Repeat steps a–e for a **second** wash, leaving supernatant in the plate to prevent the beads from overdrying.

Amplicon Indexing

This step amplifies the tagmented amplicons using a PCR program and adds unique dual indexes and additional adapter sequences required for sequencing cluster generation. The unique dual indexes include 10 base pair Index 1 (i7) adapters and 10 base pair Index 2 (i5) adapters.

Consumables

- EPM (Enhanced PCR Mix)
- Index adapters
- Nuclease-free water
- **[1-24 samples]** 1.7 ml tube
- **[24-48 samples]** 15 ml tube
- 96-well PCR plate

About Reagents

- Index adapter plates
  - Do not add samples to the index plate wells.
  - Index plate wells are considered single use and should not be reused.

Preparation

1. Prepare the following consumables:
   - EPM—Invert to mix, and then keep on ice.
   - Index adapters—Vortex to mix, and then centrifuge at 1000 × g for 1 minute.
2. Open each prepared index adapter plate seal as follows.
   - **[48 samples]**: Align a new 96-well PCR plate above the index adapter plate, and then press down to puncture the foil seal. Discard the PCR plate.
   - **[< 48 samples]**: Pierce the foil seal on the index adapter plate with a new pipette tip for each well. Pierce wells for only the number of samples to be processed.
3. Save the following IMAP-Flu TAG PCR program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 72°C for 3 minutes
   - 98°C for 3 minutes
   - 7 cycles of:
     - 98°C for 20 seconds
     - 60°C for 30 seconds
     - 72°C for 1 minute
   - 72°C for 3 minutes
   - Hold at 10°C

Procedure

1. In the appropriately sized tube, combine the following volumes to prepare PCR Master Mix. Multiply each volume by the number of samples. Reagent overage is included to account for small pipetting errors.
   - EPM (24 µl)
   - Nuclease-free water (24 µl)
2. Vortex PCR Master Mix to mix.

3. Keep the TAG1 plate on the magnetic stand and remove TWB remaining from the *Post Tagmentation Clean Up* on page 15 procedure.
4. Use a 20 µl pipette to remove any remaining TWB.
5. Remove the TAG1 plate from the magnetic stand.
6. Add 40 µl PCR Master Mix to each well.
7. Add 10 µl index adapters to each well of the PCR plate.
8. Seal and shake at 1600 rpm for 1 minute.
9. If liquid is visible on the seal, centrifuge at 500 × g for 1 minute.
10. Inspect to make sure beads are resuspended. To resuspend, set pipette to 35 µl with the plunger down, and then slowly pipette to mix.
11. Place on the preprogrammed thermal cycler and run the IMAP-Flu TAG PCR program.

Pool and Clean Up Libraries

This step combines libraries from each 96-well sample plate into one 1.7 ml tube. Libraries with optimal size are then bound to magnetic beads. Fragments that are too small or too large are washed away.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- EtOH (absolute ethanol)
- 1.7 ml tube (2 per 96-well sample plate)

About Reagents

- IPB
  - Vortex before each use.
  - Vortex frequently to make sure the beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. Prepare the following consumables:
   - IPB—Vortex to mix.
   - RSB—Vortex and invert to mix.
   - 80% EtOH—Prepare 2.5 ml 80% EtOH from absolute EtOH for each tube of pooled libraries.
2. Label a new 1.7 ml tube Pooled IPB.

Procedure

1. Centrifuge the TAG1 plate at 500 × g for 1 minute.
2. Place on the magnetic plate stand and wait until the liquid is clear (~3 minutes).
3. Transfer 5 µl supernatant from each well of the TAG1 plate into the Pooled IPB tube.
4. Vortex the Pooled IPB tube to mix, and then centrifuge briefly.
5. Vortex IPB to resuspend.
6. Add IPB using the resulting volume of Pooled IPB tube volume multiplied by 0.9.
   For example, for 48 samples add 216 µl IPB to each tube.
7. Vortex to mix.
8. Incubate at room temperature for 5 minutes.
9. Centrifuge briefly.
10. Place on the magnetic tube stand and wait until the liquid is clear (~5 minutes).
11. Remove and discard all supernatant.
12. Wash beads as follows.
    a. Keep on the magnetic stand and add 1000 µl fresh 80% EtOH to each tube.
    b. Wait 30 seconds.
    c. Remove and discard all supernatant.
13. Wash beads a **second** time.
14. Use a 20 µl pipette to remove all residual EtOH.
15. Add 55 µl RSB.

    Due to library yield excess, the RSB volume does not impact batches with a small number of samples.

16. Vortex to mix, and then centrifuge briefly.
17. Incubate at room temperature for 2 minutes.
18. Place on the magnetic tube stand and wait until the liquid is clear (~2 minutes).
19. Transfer 50 µl supernatant from each Pooled IPB tube to a new microcentrifuge tube.

SAFE STOPPING POINT

If you are stopping, cap the tube and store at -25°C to -15°C for up to 30 days.

Quantify and Normalize Libraries

1. Analyze 2 µl library pool using a Qubit dsDNA HS Assay kit.
   If libraries are outside the standard range, dilute to 1:10 concentration and analyze again.
2. Calculate the molarity value using the following formula.

- Use 500 bp as the average library size if unable to analyze the library size using the Bioanalyzer or Tapestation.

$$\frac{Library\ concentration\ ng/\mu l}{660\ \frac{g}{mol}\ x\ average\ library\ size\ (bp)} x10^6 = Molarity\ (nM)$$

3. Dilute each library pool to a minimum of 30 µl at a normalized concentration 4 nM using RSB.

Pool and Dilute Libraries

After diluting to the starting concentration of 4 nM (or the recommended starting concentration for the sequencing system), libraries are ready to be denatured and diluted to the final loading concentration.

Calculate the number of samples per run based on the number of reads per sample required and the flow cell type. For example, 0.5 M clusters and 1 M total paired-end reads per sample (0.5 M read-pairs) is a standard starting point. Challenging or diluted samples may require more reads, typically >2 M total paired-end reads per sample.

Transfer the designated volume of normalized libraries containing the appropriate index adapter sets to a new microcentrifuge tube for each number of samples. The following table lists an example at 1 M total paired-end reads.

For multiple normalized pools, combine the designated volume of each normalized pool in the tube. This process produces a final pool of samples diluted to a starting concentration of 4 nM. Do not combine pools with the same index adapter set. If additional indexes are needed, use Unique Dual Index Plate A (catalog #20026121) or Plate C (catalog #20043019).

Table 10 Normalized Pool Volumes and Sample Numbers for Denature and Dilution by Instrument using 0.5 M clusters and 1 M total paired-end reads/sample

Flow Cell Type	Volume of Normalized Libraries Used per Run (µl)	Samples per Final Pool of Normalized Libraries	Samples per Flow Cell
iSeq 100 v1 or v2	2	8	8
MiSeq i100 10M	4	10	10
MiSeq v2	5	30	30
MiSeq v3	5	48	48
MiSeq i100 25M	5	48	48
MiniSeq HO	25	48	48
NextSeq 500/550 HO	25	384*	384*
NextSeq 1000/2000 P2	25	384*	384*

Flow Cell Type	Volume of Normalized Libraries Used per Run (µl)	Samples per Final Pool of Normalized Libraries	Samples per Flow Cell
NovaSeq 6000 SP	25	384*	384 per lane, 768 per flow cell
NovaSeq 6000 S4	25	384*	384 per lane, 1536 per flow cell

* Multiplexing 384 samples requires (8) Illumina Microbial Amplicon Prep—Influenza A/B kits and (4) IDT for Illumina DNA/RNA UD Indexes (Set A, B, C, and D). These index plates must be used in place of the Illumina Unique Dual Indexes, LT that come with these library prep kits.

1. Follow the denature and dilute instructions for your sequencing system to dilute to the final loading concentration.
   Refer to the Illumina support site for more information on diluting to the final loading concentration.
2. Use the following loading concentrations for your sequencing system.

   Table 11 Loading Concentrations by Instrument

Flow Cell Type	Starting Concentration (nM)	Final Loading Concentration (pM)
iSeq 100 v1 or v2	4	75
MiSeq i100 10M	4	80
MiSeq v2	4	10
MiSeq v3	4	12
MiniSeq HO	4	1.2
NextSeq 500/550	4	1.4
NextSeq 1000/2000 P2	4	1000
NovaSeq 6000 SP	4	100
NovaSeq 6000 SP	4	100

   Adjustments to final loading concentration should follow the denature and dilute instructions for your sequencing system.

Resources and References

The Illumina support site provide additional resources. Always check support pages for the latest versions.

Revision History

Document #	Date	Description of Change
200044077 v02	September 2026	• Updated DNA/RNA Prep, Tagmentation Beads box name. • Updated formatting. • Added guidance for additional sequencers. • Added reagent mixing guidance to protocol steps. • Updated catalog numbers for index plates. • Standardized thermal cycler specifications.
200044077 v01	October 2023	• Added user-supplied equipment. • Added procedural and volume corrections to protocol steps.
200044077 v00	September 2023	Initial release.

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