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Illumina Microbial Amplicon Prep

Reference Guide

ILLUMINA PROPRIETARY
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Overview

This resource explains how to prepare up to 48 uniquely indexed targeted genomic libraries from DNA, RNA, or total nucleic acid using the Illumina Microbial Amplicon Prep (IMAP) workflow. This kit enables targeted sequencing (whole genome or select regions) of viruses and other microbes. Design your amplicon primer sets for your target(s) of interest and use those primer sets with the kit. The result is a targeted library from DNA, RNA, or total nucleic acid for sequencing on Illumina systems. Results may vary depending on the targets and are not guaranteed.

IMAP offers:

- Preparation of ≤ 48 unique dual-indexed libraries
- Scalable library prep from 1–48 samples
- Generation of sequence-ready libraries in < 9 hours
- Flexible nucleic acid inputs, RNA or DNA, depending on the nature of the target(s)
- Compatibility with user-designed target primer sets

Input Recommendations

The following section provides input recommendations.

IMAP supports the following four general input sample types:

- RNA only—Enter the workflow at Protocol step “Anneal RNA”.
- DNA only—Enter the workflow at Protocol step “Amplicon PCR”.
- Total Nucleic Acid—Have separate DNA and RNA isolates from each sample.
  - Isolated RNA enters the workflow at Protocol step “Anneal RNA”.
  - Isolated DNA enters the workflow at Protocol step “Amplicon PCR”, and is pooled with the cDNA from its corresponding sample.
- Total Nucleic Acid—Input is co-purified DNA and RNA.
  - Total Nucleic acid enters the workflow at Protocol step “Anneal RNA”.
  - May result in proportionally fewer reads from RNA than inputting separate DNA and RNA isolates per sample. Identify the optimal method for your application.

Assess Nucleic Acid Sample Purity

IMAP supports nucleic acid 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. Due to the variety and challenging nature of many sample types, it may not be possible to reach the purity listed in the following table. If you experience difficulties in making your

targeted amplicon libraries with nucleic acids that do not meet these recommendations, it may be beneficial to clean up the samples and repeat your library preparation.

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 and genomic DNA with 260/280 absorbance ratio values of 2.0–2.2 for RNA and 1.8–2.0 for DNA, 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	DNA
A260/280	2.0–2.2	1.8–2.0
A260/230	2.0–2.2	2.0–2.2

Input Amount

Given the highly variable target abundance in sample types used with this technology, Illumina recommends adding the maximum volume of input nucleic acid permitted in this protocol 8.5 µl for RNA or total nucleic acid, or 5 µl for isolated DNA. Optimize the input concentration by decreasing / diluting the sample, or increasing using various methods designed to concentrate nucleic acid (use may need to be optimized). When using purified RNA as the only input with this kit, Illumina recommends using a DNase treatment step during RNA purification.

Purification Kits

Many commercially available DNA and 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.

Target Primer Design

The IMAP kit does not provide target-specific primers. You are responsible for the design and sourcing of your target primers. You can use primer sets that are available to the research community. Amplicon lengths of 400 base pairs are recommended. Longer amplicons may be necessary with some targets.

- A program (such as Primalscheme), for primer design for tiling across adjacent amplicons, for applications such as whole genome viral sequencing is required. The program must design sets of primers that cover the entire target input sequence, with amplicons arranged end to end across the target. Use each primer set designed by the program to generate two amplicon pools, and combine at the tagmentation step. This is the method used with ARTIC primers for SARS-CoV-2 sequencing.

  Assay performance is dependent upon input of representative genome reference(s) into Primalscheme. Primer-template mismatch causes amplicon dropout, affecting viral genome coverage.

- You can use other primer design programs for non-tiling targeted sequencing applications. Follow the recommended design parameters for the chosen design program. Design the primers for a multiplex PCR assay.
- Aim for primer annealing temperatures of 63–65°C and try to keep the predicted temperatures consistent across amplicon primer pairs in a set. 63°C is the recommended starting temperature. Depending on the target, adjust the desired annealing temperature of the primers to optimize PCR amplification.
- Dilute the primer pools to 10 µM. Use 4.3 µl of each primer pool per sample or a total of 207 µl of each pool for 48 total samples.

Consumables and Equipment

The IMAP 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

Completing the IMAP protocol requires the following library prep reagents.

Library Prep Kit Option	Catalog #
Illumina Microbial Amplicon Prep (48 samples)	20097857

Reagent Kit Contents

Each library prep reagent kit comprises four 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
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

User-Supplied Consumables and Equipment

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

Consumables

Consumables	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

Consumables	Supplier
1.7 ml LoBind microcentrifuge tubes	Eppendorf, catalog # 022431021
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
Dynamag-2 Magnet	Thermo Fisher, catalog 12321D
Magnetic Stand	Thermo Fisher, catalog # AM10027

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 kit.

The following protocol has been tested with multiple viral targets including SARS-CoV-2, Mpox (formerly monkeypox), and Dengue virus. The protocol is the recommended starting point for developing a new protocol with new targets and primer sets.

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.

Input Types

- RNA-only—Start the protocol at the Anneal RNA step.
- DNA-only—Start the protocol at the Amplicon PCR step.
- RNA and DNA, separate isolates—For inputs purified separately from the same starting samples, start the protocol at the Anneal RNA step using the RNA input. At the Amplicon PCR step, combine the cDNA and purified DNA. This strategy may produce more balanced coverage of both RNA and DNA targets compared to co-isolated RNA and DNA.
- RNA and DNA, co-isolated—Start protocol at the Anneal RNA step.

The protocol provides reminders and specific changes necessary when using these input types. Some steps may require optimization, and possible optimization points are highlighted throughout the protocol.

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 or RNA/DNA co-isolate samples from storage.
2. Remove the reagents and prepare as follows.

Table 5 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EPH3	Box 3	Thaw at room temperature.
FSM	Box 3	Thaw at room temperature.
RVT	Box 3	Thaw on ice.

Anneal RNA

Start with this section if using **RNA** sample inputs.

During this process, random hexamers are annealed to the extracted RNA to prepare for cDNA synthesis. The random hexamers are contained in EPH3. In most cases, random hexamers are the best choice for first strand cDNA synthesis. There may be cases where different reverse transcriptase (RT) primers, such as target specific primers, may perform better. However, in some cases, other RT primers

(such as target-specific primers), may perform better. If random hexamers are not used in the reaction, it will affect total salt concentrations. Carefully analyze the targets and goals of your experiment to determine the best RT primers to use, or consult with the Illumina Technical Support team.

Consumables

- EPH3 (Elution Prime Fragment 3HC Mix)
- 96-well PCR Plate
- Microseal 'B' adhesive seals

About Reagents

- EPH3—Vortex to mix before each use.

Preparation

1. Prepare the following consumables:
   - EPH3—Vortex to mix.
2. Save the following IMAP Anneal program on the thermal cycler:
   - Choose the preheat lid option at 105°C
   - Set the reaction volume to 17 µl
   - 65°C for 3 minutes
   - Hold at 4°C
3. Label a new PCR plate CDNA1.

Procedure

1. Add 8.5 µl EPH3 to each well.
2. Add 8.5 µl RNA or RNA/DNA co-isolate sample to each well.
3. Seal and shake at 1600 rpm for 1 minute.
4. Centrifuge at 1000 × g for 1 minute.
5. Place on the preprogrammed thermal cycler and run the IMAP Anneal program.

Synthesize First Strand cDNA

This step reverse transcribes the RNA fragments primed with random hexamers into first strand cDNA using reverse transcriptase.

Consumables

- FSM (First Strand Mix)

- RVT (Reverse Transcriptase)
- 1.7 ml microcentrifuge tubes (1 per 96-well sample plate)
- Microseal 'B' adhesive seal

Preparation

1. Prepare the following consumables:
   - FSM—Invert to mix.
   - RVT—Invert to mix before each use.
2. Save the following IMAP FSS program on the thermal cycler:
   - Choose the preheat lid option at 105°C
   - Set the reaction volume to 25 µl
   - 25°C for 5 minutes*Optimization Option
   - 50°C for 10 minutes*Optimization Option
   - 80°C for 5 minutes
   - Hold at 4°C

   *Optimization Option

   Optimize the reverse transcription steps by changing the temperature and incubation time. 50°C is the maximum recommended temperature for this RT enzyme. cDNA synthesis may be improved at lower temperatures (~42°C).

Procedure

1. In a 1.7 ml tube, combine the following volumes to prepare FSM + RVT mixture. Multiply each volume by the number of samples.
   - FSM (9 µl)
   - RVT (1 µl)

   Reagent overage is included to account for small pipetting errors.
2. Add 8 µl FSM + RVT mixture to each well of the CDNA1 plate.
3. Seal and shake at 1600 rpm for 1 minute.
4. Centrifuge at 1000 × g for 1 minute.
5. Place on the preprogrammed thermal cycler and run the IMAP FSS program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Prepare for Protocol

1. Remove DNA samples and/or cDNA (CDNA1 plate) from storage.
2. Remove the reagents and prepare as follows.

Table 6 -25°C to -15°C Storage

Reagent	Box Name	Instructions
IPM	Box 3	Thaw on ice.
PP1	User-designed and sourced.	Thaw on ice.
PP2	User-designed and sourced.	Thaw on ice.

Amplicon PCR

DNA samples typically enter the workflow at this step. Start the workflow here if using **DNA only**.

If using seperate isolates of **DNA and RNA** from each sample, the purified DNA from each sample is added to its corresponding cDNA (formerly RNA) sample at this stage.

This step uses the custom primer pool(s) to amplify targets of interest from each sample to produce the amplicons that will be tagmented.

This portion of the protocol is designed to use two primer pools to tile across a genome or large target region. You can also use this protocol with one primer pool.

Consumables

- IPM (Illumina PCR Mix)
- Primer Pool 1 (PP1) (User-designed and sourced)
- Primer Pool 2 (PP2) (User-designed and sourced)
- Nuclease-free water
- 15 ml tube
- 96-well PCR plates (2)
- Microseal 'B' adhesive seal

Preparation

1. Prepare the following consumables:
   - PP1—Keep on ice until use.

- PP2—Keep on ice until use.
- IPM—Invert to mix. Keep on ice until use.

2. Save the following IMAP PCR program on the thermal cycler:
   - Choose the preheat lid option at 105°C
   - Set the reaction volume to 25 µl
   - 98°C for 3 minutes
   - 35 cycles of:
     - 98°C for 15 seconds
     - 63°C for 5 minutes*Optimization Option
   - Hold at 4°C

   *Optimization Option

   Optimize by changing the temperature and incubation time depending on your targets and amplicons. For example, decrease the combination 63°C anneal/extension temperature to better match the annealing temperatures of the PCR primer pools to create a true annealing step in the cycle. Alternately, insert an additional extension step at 72°C into the cycle.
3. Label two new PCR plates PP1 and PP2.
   The plates represent two separate PCR reactions on each sample in the CDNA1 plate. Alternatively, you can set up both sets of PCR reactions in a single PCR plate.

Procedure

1. In two 1.7 ml tubes, combine the following reagents to prepare PCR 1 Master Mix and PCR 2 Master Mix. Multiply each volume by the number of samples.
   Reagent overage is included to account for small pipetting errors.

Reagent	PCR 1 Master Mix (µl)	PCR 2 Master Mix (µl)
IPM	15	15
PP1	4.3	N/A
PP2	N/A	4.3
Nuclease-free water	4.7	4.7

2. Vortex or manually pipette to mix.
3. Add 20 µl PCR 1 Master Mix to each well of the PP1 plate.
4. Proceed as follows:

- **[RNA only]**: Add 5 µl cDNA from each well of the CDNA1 plate to the corresponding well of the PP1 plate.
- **[DNA only]**: Add 5 µl purified DNA from each sample to a well of the PP1 plate.
- **[RNA and DNA]**: Add 2.5 µl cDNA from each well of the CDNA1 plate to the corresponding well of the PP1 plate, followed by 2.5 µl of purified DNA from the same sample to the corresponding well of the PP1 plate. Each well of the PP1 plate should contain 2.5 µl of cDNA and 2.5 µl of DNA from the same starting sample, plus the PCR 1 Master Mix.

5. Add 20 µl PCR 2 Master Mix to each well of the PP2 plate.
6. Proceed as follows:
   - **[RNA only or co-purified DNA/RNA]**: Add 5 µl cDNA from each well of the CDNA1 plate to the corresponding well of the PP2 plate.
   - **[DNA only]**: Add 5 µl purified DNA from each sample to a well of the PP2 plate.
   - **[Separate RNA and DNA isolates]**: Add 2.5 µl cDNA from each well of the CDNA1 plate to the corresponding well of the PP2 plate, followed by 2.5 µl of purified DNA from the same sample to the corresponding well of the PP2 plate. Each well of the PP2 plate should contain 2.5 µl of cDNA and 2.5 µl of DNA from the same starting sample, plus the PCR 2 Master Mix.
7. Seal and shake at 1600 rpm for 1 minute.
8. Centrifuge at 1000 × g for 1 minute.
9. Place in the preprogrammed thermal cycler and run the IMAP PCR program.

SAFE STOPPING POINT

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

Prepare for Protocol

1. Remove PP1 and PP2 plates 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	Bring to room temperature.
TWB	Box 2	Bring to room temperature.

Table 9 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EPM	Box 3	Thaw on ice.
Index adapters	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 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 PP1 and PP2 were 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 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. Label a new PCR plate TAG1.
2. Combine PP1 and PP2 as follows.
   a. Transfer 10 µl from each well of the PP1 plate to the corresponding well of the TAG1 plate.
   b. Transfer 10 µl from each well of the PP2 plate to each well of the TAG1 plate containing starting sample from PP1.

   If you are only using one primer pool, add 10 ul of nuclease-free water in place of PP2, to total 20 µl.

3. 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)
4. Pipette the Tagmentation Master Mix thoroughly to mix.
5. Add 30 µl Tagmentation master mix to each well of the TAG1 plate.
6. Seal and shake at 1600 rpm for 1 minute.
7. Place on the preprogrammed thermal cycler and run the IMAP 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
- 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 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 15 ml 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 17 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 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 400 bp as the average library size.

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

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 reads per sample is a standard starting point. Challenging or diluted samples such as waste water may require more reads, typically >2 M 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 0.5 M 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 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

Flow Cell Type	Volume of Normalized Libraries Used per Run (µl)	Samples per Final Pool of Normalized Libraries	Samples per Flow Cell
MiSeq i100 25M	5	48	48
MiniSeq HO	25	48	48
NextSeq 500/550 or 550Dx HO	25	384*	384*
NextSeq 1000/2000 P2	25	384*	384*
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 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 or 550Dx HO	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.

Prepare for Sequencing

Download a sample sheet template specific to your sequencing system (available on the Illumina support site), to help create a sample sheet for your run.

Set Up Sequencing Run

To set up the run, follow the Illumina support site documentation specific to your sequencing system.

Analysis Software

After sequencing completes, analyze your data in BaseSpace Sequence Hub with the DRAGEN Targeted Microbial App. Contact Illumina customer support for more information.

Resources and References

The following sections provide primer and genome file format specifications. The Illumina support site provide additional resources. Always check support pages for the latest versions.

Primer Definition File Format Specification

If the primer binding site coordinates are known, provide a BED file that uses the following four columns (as a minimum):

- `chrom`
- `chromStart`
- `chromEnd`
- `primerName`

If the primer binding site coordinates are not known, provide primer sequences in any one of the following formats:

Format	# of Columns	Columns	Notes
Modified PrimalScheme BED	7 or more	chrom, chromStart, chromEnd, primerName, pool, strand, sequence	Coordinates are re-predicted based on primerName, strand and sequence. Any columns after the 7th are ignored.
tntBLAST	3	ampliconName, forwardSequence, reverseSequence	ampliconName must be unique
Grubaugh lab	3	primerName, sequence, pool	Not applicable

Formatting Rules

General

- All text is case sensitive.
- Any line starting with '#' is ignored. This can be used to add a header line with column names.
- Every line must have the same number of columns and format (except those starting with '#').
- Any number of spaces or tabs can separate the columns. A value within a single column should not have any space.

BED format

- Per standard BED conventions, sequence coordinates are given as 0-based, half-open intervals, such that the `chromStart` field (2nd column) contains the first nucleotide in the primer binding site and the last nucleotide in the primer binding site is the value in the `chromEnd` field (3rd column) minus 1.
- `chrom` field must contain a sequence identifier matching a FASTA file header. The FASTA file contains a sequence relative to the coordinates.
- Multiple sequence identifiers (`chrom`) are permitted within one file (modified from standard PrimalScheme format).

Primer name

- `primerName` must be unique and must encode the following items:
  - The name of the amplicon for which the primer is designed.
  - The direction tag indicating which side of the amplicon (left or right) the primer belongs to.
  - An optional indicator if the primer is an 'alternative' primer for that amplicon.
- In addition to _LEFT and _RIGHT, _L and _R are permitted as direction tags in `primerName` (modified from standard PrimalScheme format). Any text after the direction tag should be separated by an underscore (_).
- Text in `primerName` before the direction tag is considered an amplicon identifier. Make sure that the text of the amplicon identifier is unique for that amplicon and that the direction tag occurs only once in `primerName`.
- Each amplicon must have at least one left and right primer (including alternative primers) associated with it.
- Alternative primers are used to bind to locations that avoid sequence variation in the default primer binding site that may disrupt hybridization. An amplicon may have an arbitrary number of alternative primers (as long as the primer name is unique), but most amplicons will have none. Alternative primers are indicated by the presence of the _alt after the direction tag in `primerName`, followed by optional text to distinguish between different alternative primers, such as a number.
- Examples of valid primer names:
  - MY_SEQUENCE_434_A_LEFT
  - virus1_L
  - amplicon_4934m_RIGHT_alt
  - amplicon_4934m_RIGHT_alt1
  - amplicon_4934m_R_altprimerB
- Examples of invalid primer names:
  - LEFT_MY_SEQUENCE_434_A
  - virus1_l

– amplicon_4934m_RIGHT_L

Following is an example of a section of a valid primer definition file. (Excerpts are from the ARTIC SARS-CoV-2 v4.1 primer definitions).

NC_045512.2	25	50	SARS-CoV-2_1_LEFT	1	+	AACAAACCAACCAACTTTCGATCTC
NC_045512.2	324	344	SARS-CoV-2_2_LEFT	2	+	TTTACAGGTTCGCGACGTGC
NC_045512.2	408	431	SARS-CoV-2_1_RIGHT	1	-	CTTCTACTAAGCCACAAGTGCCA
NC_045512.2	644	666	SARS-CoV-2_3_LEFT	1	+	GTAATAAAGGAGCTGGTGGCCA
NC_045512.2	705	727	SARS-CoV-2_2_RIGHT	2	-	ATAAGGATCAGTGCCAAGCTCG
NC_045512.2	944	966	SARS-CoV-2_4_LEFT	2	+	GTGTATACTGCTGCCGTGAACA
NC_045512.2	1017	1044	SARS-CoV-2_3_RIGHT	1	-	GCCAATTTAATTTCAAAAGGTGTCTGC
NC_045512.2	1245	1266	SARS-CoV-2_5_LEFT	1	+	TGAAACTTCATGGCAGACGGG
NC_045512.2	1337	1362	SARS-CoV-2_4_RIGHT	2	-	ACAACAGCATTTTGGGGTAAGTAAC
NC_045512.2	1540	1562	SARS-CoV-2_6_LEFT	2	+	CGTGCTAGCGCTAACATAGGTT
NC_045512.2	1851	1875	SARS-CoV-2_7_LEFT	1	+	ACTGAGTCCTCTTTATGCATTTGC
NC_045512.2	1925	1948	SARS-CoV-2_6_RIGHT	2	-	AACACGCACAGAATTTTGAGCAG
NC_045512.2	2154	2180	SARS-CoV-2_8_LEFT	2	+	GCTTGAAGAGAAGTTTAAGGAAGGTG
NC_045512.2	2228	2250	SARS-CoV-2_7_RIGHT	1	-	CCACCGACAATTTCACAAGCAC
NC_045512.2	2483	2508	SARS-CoV-2_9_LEFT	1	+	TCTTCTTAGAGGGAGAAACACTTCC
NC_045512.2	2544	2571	SARS-CoV-2_8_RIGHT	2	-	GGTTGTTCTAATGGTTGTAAATCACCA
NC_045512.2	2780	2813	SARS-CoV-2_10_LEFT_alt1	2	+	TGAATATCACTTTTGAACTTGATGAAAGGATTG
NC_045512.2	2826	2850	SARS-CoV-2_10_LEFT	2	+	TGAGAAGTGCTCTGCCTATACAGT
NC_045512.2	2861	2885	SARS-CoV-2_9_RIGHT	1	-	CACAGGCGAACTCATTTACTTCTG
NC_045512.2	3078	3102	SARS-CoV-2_11_LEFT	1	+	AGAAGAGTTTGAGCCATCAACTCA
NC_045512.2	3156	3177	SARS-CoV-2_10_RIGHT_alt1	2	-	GGTTGAAGAGCAGCAGAAGTG
NC_045512.2	3183	210	SARS-CoV-2_10_RIGHT	2	-	TCATCTAACCAATCTTCTTCTTGCTCT

Genome Definition File Format Specification

The genome definition file is a BED-like tab-separated value (*.tsv) file with no header row, consisting of the following columns. Coordinates are 0-based, half-open intervals according to the BED standard.

Column name	Description
chrom	The sequence identifier (accession) of the reference genome as defined by the FASTA header text between the leading > character and the first whitespace character.
chromStart	The first genomic coordinate of the reference genome (usually 0).

Column name	Description
chromEnd	The last genomic coordinate of the reference genome (usually the length of the sequence).
genomeName	The human-readable name for this genome used in reports.
segmentName	For multi-segment or genomes, the human-readable identifier for this segment, beginning with "Segment". For non-segmented viruses, this field should be "Full".
genomeGroup	The general category of the genome, used for report masking (choosing to report only some categories of organisms).
nextcladeDataset	The identifier of the NextClade dataset, if any, to use for phylogenetic analysis of the consensus genomes generated from this reference genome.

The following is an example of a genome definition file:

NC_003310.1	0	196858	MPXV	Full	Monkeypox	MPXV
NC_063383.1	0	197209	MPXV	2	Monkeypox	MPXV
MH539865.1	0	6562	Monongahela hantavirus	1	Hantavirus	N/A
MH539866.1	0	3671	Monongahela hantavirus	1	Hantavirus	N/A
MH539867.1	0	2081	Monongahela hantavirus	2	Hantavirus	N/A
NC_006572.1	0	7271	Mopeia Lassa virus reassortant 29	2	Lassa fever virus	N/A
NC_006573.1	0	3402	Mopeia Lassa virus reassortant 29	1	Lassa fever virus	N/A

Revision History

Document #	Date	Description of Change
200039808 v01	September 2026	• Updated DNA/RNA Prep, Tagmentation Beads box name. • Reformatted to align with Illumina Microbial Amplicon Prep—Influenza A/B Product Documentation. • Clarified the descriptions of potential sample input configurations. • Added guidance for additional sequencers. • Added reagent mixing guidance to protocol steps. • Updated catalog numbers for index plates. • Standardized thermal cycler specifications.
200039808 v00	July 2023	Initial release.

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