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Illumina RNA Prep with Enrichment, (L) Tagmentation

Product Documentation

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Table of Contents

Overview

The Illumina RNA Prep with Enrichment, (L) Tagmentation kit pairs with an index kit and enrichment panel to generate enriched libraries for dual-indexed, paired-end sequencing. Reverse transcription converts RNA into complementary DNA (cDNA), which is then tagmented and amplified to add indexes and other adapters. The resulting libraries are normalized for one- or three-plex enrichment and further amplification.

The Illumina RNA Prep with Enrichment, (L) Tagmentation 16 sample kit (20040536) includes reagents for preparing and enriching 16 libraries (16, 1-plex enrichment reactions). The Illumina RNA Prep with Enrichment, (L) Tagmentation 96 sample kit (20040537) includes reagents for preparing and enriching 96 libraries (32, 3-plex enrichment reactions). Enrichment probe panel, purification beads and index adapters must be purchased separately.

Sequence-specific biotinylated probes combine with magnetic beads to capture regions of interest. The captured sequences are washed, eluted, and amplified to generate copies of the enriched library. A limited-cycle PCR program exponentially amplifies the enriched fragments, copying each fragment to increase the amount of library.

The kit offers the following features:

- Tagmentation with Enrichment Bead-Linked Transposomes (EBLTL) to create larger inserts
- Unique dual (UD) indexing with Illumina DNA/RNA UD Indexes
- High-quality sequencing data from a range of input, 10–100 ng total RNA

Input Recommendations

The protocol is optimized for the following input sample types:

- 10–100 ng of purified total RNA
- 20–100 ng RNA input from degraded or FFPE samples (DV200 ≥ 36.5)

Lower input amounts and lesser quality can reduce library yield.

Include a DNase treatment with the RNA isolation method. The DNase treatment ensures sample purity and accurate quantification. Before starting the protocol, quantify the total RNA using standard methods and assess quality using a fragment analysis method.

For infectious disease and microbiology panels including Respiratory Virus Enrichment Kit (RVEK), and Respiratory Pathogen ID/AMR Oligo Panel (RPIP):

- The panels include both DNA and RNA targets and use an input of total nucleic acid or an equal pool by volume of separately eluted DNA and RNA from the same sample. Do not perform DNase steps as part of the protocol if DNA is included as part of the input (total nucleic acid or mix of separately extracted RNA and DNA).

- Achieving the recommended input of 10–100 ng of total nucleic acid is unlikely from low biomass samples. In this case, a standard volume of 7.5 µl nucleic acid eluate (total nucleic acid or mix of separately extracted RNA and DNA) is recommended.

Enrichment Panel Recommendations

Supplemental enrichment panels can be designed to add or boost coverage of the following fixed and customizable biotinylated oligo enrichment panels.

- Illumina Custom Enrichment Panel v2 (ICEPv2)
- Respiratory Virus Enrichment Kit (RVEK) using Respiratory Virus Oligo Panel v2 (RVOPv2)
- Illumina Exome Panel (CEX)
- Illumina Respiratory Pathogen ID/AMR Enrichment Panel Kit (RPIP)
- Pan-Coronavirus Panel (Pan-CoV)

The Illumina Support Center provides additional product-specific resources.

DesignStudio Assay Design Tool supports assay designs for human hg19 and hg38 genome assemblies. Contact Illumina Technical Support for non-human custom enrichment panel designs.

Consumables and Equipment

This section lists all components included in the reagent kit, with storage conditions. This section also details the ancillary consumables, equipment, and other prerequisites needed to complete the protocol.

The protocol has 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

Make sure that you have all reagents identified in this section before starting library prep. The protocol requires one Illumina RNA Prep with Enrichment, (L) Tagmentation kit, one panel, and at least one Illumina DNA/RNA UD Indexes set. Combine all four index adapter sets to index 384 libraries.

- The required library preparation kits provide reagents for denaturing, cDNA synthesis, library prep, and enrichment in a 16-sample 1-plex workflow, or 96-sample 3-plex workflow.
- The enrichment panels provide application-specific oligos.
- The index sets provide premixed Index 1 (i7) and Index 2 (i5) adapters.

Illumina Purification Beads (IPB) are also required. These reagents are not included in the Illumina kits and must be purchased separately. Refer to *User-Supplied Consumables and Equipment* on page 7 for the IPB quantity requirements and catalog numbers.

Component	Name	Catalog #
Library prep reagents	Illumina RNA Prep with Enrichment, (L) Tagmentation (16 samples)[1]	20040536
	Illumina RNA Prep with Enrichment, (L) Tagmentation (96 samples)[2]	20040537
Index adapters	Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 indexes, 96 samples)	20091654
	Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 indexes, 96 samples)	20091656
	Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 indexes, 96 samples)	20091658
	Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 indexes, 96 samples)	20091660
Enrichment Panels	Respiratory Virus Oligo Panel v2	20044311
	Illumina Exome Panel (CEX)	20020183
	Pan-Coronavirus Panel	20088155
	Respiratory Virus Enrichment Kit Set A (96 samples)	20100469
	Respiratory Virus Enrichment Kit Set B (96 samples)	20100470
	Respiratory Virus Enrichment Kit Set C (96 samples)	20100471
	Respiratory Virus Enrichment Kit Set D (96 samples)	20100472
	Illumina Custom Enrichment Panel v2 (32 µl, 120 bp)	20073953
	Illumina Custom Enrichment Panel v2 (384 µl, 120 bp)	20073952
	Illumina Custom Enrichment Panel v2 (1536 µl, 120 bp)	20111339
Respiratory Pathogen ID/AMR Enrichment Kit (RVEK uses the RVOPv2 panel)	Respiratory Pathogen ID/AMR Oligo Enrichment Kit (RUO) A	20047050
	Respiratory Pathogen ID/AMR Oligo Enrichment Kit (RUO) B	20046969
	Respiratory Pathogen ID/AMR Oligo Enrichment Kit (RUO) C	20047051
	Respiratory Pathogen ID/AMR Oligo Enrichment Kit (RUO) D	20047052

[1]Kit includes reagents for 16 one-plex enrichment reactions.
[2]Kit includes reagents for 32 three-plex enrichment reactions.

Reagent Kit Contents

Illumina cDNA Synthesis, Store at -25°C to -15°C

Tube Quantity		Reagent	Description
16 Samples	96 Samples		
1	4	EPH3	Elute, Prime, Fragment High Concentration Mix
1	4	FSA	First Strand Synthesis Act D Mix
1	2	RSB	Resuspension Buffer This RSB is stored frozen.
1	1	RVT	Reverse Transcriptase
1	4	SMM	Second Strand Marking Master Mix

Illumina DNA/RNA Prep Tagmentation PCR Reagents, Store at -25°C to -15°C*

Tube Quantity		Reagent	Description
16 Samples	96 Samples		
2	4	EPM	Enhanced PCR Mix
1	4	TB1	Tagmentation Buffer 1

*Shipped at 2°C to 8°C. Promptly store at the indicated temperature to ensure proper performance.

Illumina RNA Fast Hyb Enrichment PCR + Buffers, Store at -25°C to -15°C*

Tube Quantity		Reagent	Description
16 Samples	96 Samples		
1	2	EE1	Enrichment Elution Buffer 1

Tube Quantity		Reagent	Description
16 Samples	96 Samples		
1	4	EEW	Enhanced Enrichment Wash
1	2	EPM	Enhanced PCR Mix
1	1	HP3	2 N NaOH
1	1	NHB2	Hyb Buffer 2+IDT NXT Blockers
1	1	PPC	PCR Primer Cocktail

*Shipped at 2°C to 8°C. Promptly store at the indicated temperature to ensure proper performance.

Illumina DNA/RNA Prep Tagmentation Beads, Store at 2°C to 8°C

Tube Quantity		Reagent	Description
16 Samples	96 Samples		
1	4	EBLTL	Enrichment Bead-Linked Transposomes
1	2	RSB	Resuspension Buffer*

*Promptly store at the indicated temperature to ensure proper performance.

Illumina RNA Fast Hyb Enrichment Beads + Buffers, Store at 2°C to 8°C

Tube Quantity		Reagent	Description
16 Samples	96 Samples		
1	1	EHB2	Enrich Hyb Buffer 2
1	1	ET2	Elute Target Buffer 2

Tube Quantity		Reagent	Description
16 Samples	96 Samples		
1	1	RSB	Resuspension Buffer* This RSB is stored cold.
1	2	SMB4	Streptavidin Magnetic Beads 4

*Promptly store at the indicated temperature to ensure proper performance.

Illumina DNA/RNA Prep Tagmentation Buffers, Store at Room Temperature*

Tube Quantity		Reagent	Description
16 Samples	96 Samples		
1	4	ST2	Stop Tagment Buffer 2
1	1	TWB	Tagmentation Wash Buffer

*Shipped at 2°C to 8°C. Promptly store at the indicated temperature to ensure proper performance.

Index Adapters and Enrichment Panels

Illumina DNA/RNA UD Indexes (96 Indexes, 96 Samples), Store at -25°C to -15°C

Quantity	Reagent	Description
1	UDP0001–UDP0096	Set A index adapter plate
1	UDP0097–UDP0192	Set B index adapter plate
1	UDP0193V3–UDP0288V3	Set C index adapter plate
1	UDP0289V2–UDP0384	Set D index adapter plate

Enrichment Panels, Store at -25°C to -15°C

Panel Name	Quantity	Reagent	Description
Illumina Exome Panel	1	CEX	Coding Exome Oligos

Panel Name	Quantity	Reagent	Description
Pan-Coronavirus Panel	1	Pan-CoV	Pan-Coronavirus Oligos
Illumina Custom Enrichment Panel v2	1	ICEPv2	Custom Panel Oligos
Respiratory Virus Oligo Panel v2 (Respiratory Virus Enrichment Kit (RVEK) uses RVOPv2 panel)	1	RVOPv2	Respiratory Virus Oligos
Respiratory Pathogen ID/AMR Oligo	1	RPIP	Respiratory Pathogen ID/AMR Oligos

User-Supplied Consumables and Equipment

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

Consumables

Consumable	Supplier/Description
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
96-well 0.8 ml polypropylene deep-well storage plate (MIDI plate)	Thermo Fisher Scientific, catalog # AB-0859
Disposable gloves, powder free	General lab supplier
Eppendorf twin.tec PCR Plate 96, semiskirted, 250 µl, PCR clean	Eppendorf, catalog # 951020303
Ethanol (EtOH), molecular biology grade (500 ml)	General lab supplier
One of the following depending on assay throughput:* • Illumina Purification Beads, 30 ml • Illumina Purification Beads, 100 ml • Illumina Purification Beads, 400 ml	Illumina, catalog #: • 20119944 • 20060057 • 20060058
Lab tape	General lab supplier
Microseal 'B' PCR Plate Sealing Film	Bio-Rad, catalog # MSB1001
Microcentrifuge tubes, 1.5 ml	General lab supplier

Consumable	Supplier/Description
Microcentrifuge tubes, 1.7 ml	General lab supplier
Microcentrifuge tubes, RNase-free, 1.7 ml	General lab supplier
One of the following kits, depending on quality analysis method: • **[Bioanalyzer System]** Agilent DNA 1000 Kit • **[TapeStation System]** D1000 ScreenTape • **[TapeStation System]** High Sensitivity D1000 ScreenTape	Agilent Technologies, catalog #: • 5067–1504 • 5067–5582 • 5067–5584
Qubit Assay Tubes	Thermo Fisher Scientific, catalog # Q32856
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific, catalog # Q32850 or Q32853
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, catalog # 89094-658 or equivalent
Ultrapure water, nuclease-free	General lab supplier

* Each sample prepared with the Illumina RNA Prep with Enrichment, (L) Tagmentation protocol requires a total of 261 µl IPB. Preparation of 16 samples requires 4.2 ml IPB. Preparation of 96 samples requires 25.1 ml IPB. Illumina recommends purchasing 30 ml IPB for 96 samples.

Equipment

Equipment	Supplier/Description
Pipettes, multichannel, 10 µl	General lab supplier
Pipettes, multichannel, 20 µl	General lab supplier
Pipettes, multichannel, 200 µl	General lab 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
Adhesive seal roller	General lab supplier
Magnetic Stand-96	Thermo Fisher Scientific, catalog # AM10027
Microplate centrifuge	General lab supplier
Microsample incubator	General lab supplier
Microsample incubator block, 1.5 ml	General lab supplier

Equipment	Supplier/Description
Quality analysis instrument, any of the following: • 2100 Bioanalyzer System* • 4150 TapeStation System • 4200 TapeStation System	Agilent Technologies, catalog #: • G2939BA* • G2992AA • G2991BA
Vortexer	General lab supplier
[Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa)	Millipore catalog # UFC503008

* End of life announced. Refer to vendor site for more information.

Thermal Cyclers

The following table lists recommended thermal cyclers or 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
• 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 the Illumina RNA Prep with Enrichment, (L) Tagmentation protocol and provides instructions for preparing and enriching libraries.

- Review the complete sequencing workflow, from sample through analysis, to ensure compatibility of products and experiment parameters.

- Confirm kit contents and make sure that you have the required consumables and equipment. For a complete list, refer to *Consumables and Equipment* on page 2.

Pooling Preparation

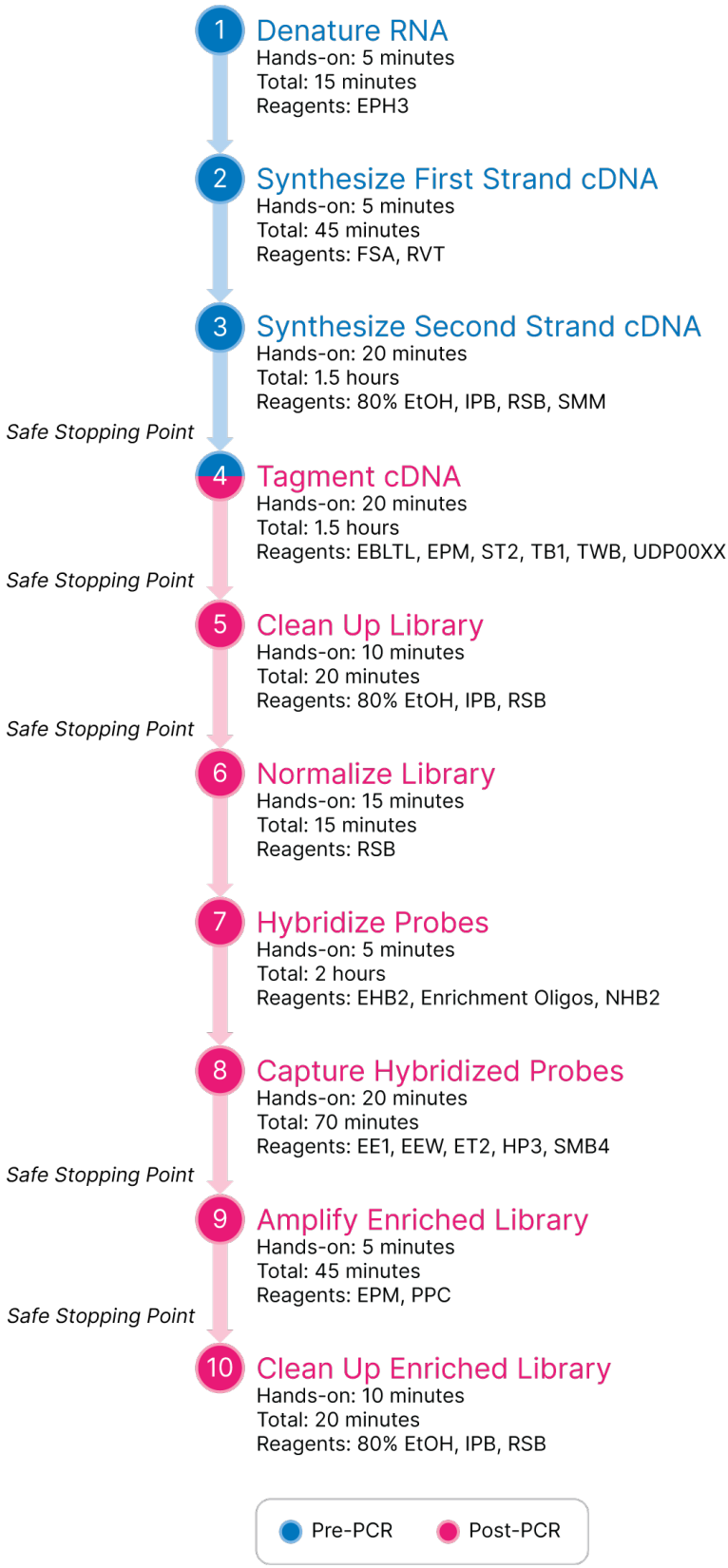
When pooling libraries, record information about your samples before starting library prep. Use a recording tool compatible with your sequencing system and libraries. For compatibility information, refer to the Enrichment panel support pages or the support pages for your system.

The protocol uses Illumina DNA/RNA UD Indexes to index libraries. These indexes add distinct Index 1 (i7) and Index 2 (i5) sequences to each end of a fragment. Each index sequence is 10 bp long.

- For strategies on forming low-plex, color-balanced pools, refer to the *Index Adapters Pooling Guide*.
- For index adapter sequences and how to record them, refer to *Illumina Adapter Sequences*.

Illumina RNA Prep with Enrichment, (L) Tagmentation Workflow

The following diagram provides an overview of the Illumina RNA Prep with Enrichment, (L) Tagmentation protocol using a single sample. Safe stopping points are marked between steps.



Tips and Techniques

Protocol Continuity

- Follow the protocol in the order described using the specified parameters.
- Avoid extended pauses until RNA is converted into double-stranded cDNA.
- Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each well***.
- Remove unused index adapter plates from the working area.

Handling Reagents and RNA

- Avoid multiple freeze-thaw cycles of input RNA.
  - You can store RNA in RNase-free water or TE buffer at -85°C to -65°C for up to one year.
  - If you must reuse the sample, aliquot 7.5 µl or less into separate tubes for single use.
- Keep thawed reagents on ice until needed. Promptly return all reagents to storage after use.
- When not in use, seal plates and close lids to limit contamination.

Handling Beads

The protocol uses more than one type of bead. Each bead has a specific technical application. Do not substitute one bead for another.

Apply the following techniques when handling beads:

- Use all beads at room temperature.
- Never use IPB or SMB4 that have been stored below 2°C.
- Aspirate and dispense beads slowly due to viscosity.
- Vortex beads frequently throughout the protocol to resuspend. Resuspended beads are evenly distributed and homogenous in color.
- If EBLTL or SMB4 beads adhere to well walls, centrifuge at 280 × g for 3 seconds, and then pipette to resuspend.
- Dispense liquid so that beads on the side of the wells are wetted.
- Dispense liquid directly onto bead pellets.
- When the plate is on the magnetic stand, do not agitate the plate or disturb the bead pellet.

- If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

Sealing the Plate

- Use Microseal 'B' adhesive seals throughout the protocol. The seals are effective at -40°C to 110°C.
- Cover the plate with the seal, and seal with a rubber roller or wedge.
- After each use, discard seals from plates.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of the first plate to the corresponding well of the second plate.

Mixing and Centrifugation

- At any step, centrifuge at 280 × g for 10 seconds to consolidate liquid or beads in the bottom of the well to prevent sample loss.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next stopping point.

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

Table 1 Room Temperature Storage

Reagent	Box Name	Instructions
IPB	Illumina Purification Beads	Use at room temperature.

Table 2 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EPH3	Illumina cDNA Synthesis	Thaw at room temperature.
FSA	Illumina cDNA Synthesis	Thaw on ice.
RSB	Illumina cDNA Synthesis	Thaw at room temperature.

Reagent	Box Name	Instructions
RVT	Illumina cDNA Synthesis	Thaw on ice.
SMM	Illumina cDNA Synthesis	Thaw on ice.

Denature RNA

This step denatures the total RNA and anneals random hexamers. The random hexamers prime the sample for cDNA synthesis.

Consumables

- EPH3 (Elute, Prime, Fragment High Concentration Mix)
- 96-well PCR plate, semiskirted
- Microseal 'B' adhesive film
- Nuclease-free ultrapure water

Preparation

1. Prepare the following consumables:
   - EPH3—Vortex to mix, and then centrifuge briefly.
2. Save the following DEN_RNA program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 17 µl
   - 65°C for 5 minutes
   - Hold at 4°C

   Total program time is ~5 minutes.

Procedure

1. In each well of a new PCR plate, dilute 10–100 ng total RNA in nuclease-free ultrapure water to a total volume of 8.5 µl.
2. Add 8.5 µl EPH3 to each well.
3. Pipette 10 times to mix.
4. Seal the plate, and then centrifuge at 280 × g for 3 seconds.
5. Place on the preprogrammed thermal cycler and run the DEN_RNA program.
6. Centrifuge at 280 × g for 10 seconds.

Each well contains 17 µl denatured RNA bound with random hexamers.

Synthesize First Strand cDNA

This step reverse transcribes the hexamer-primed RNA fragments to produce first strand complementary DNA (cDNA).

Consumables

- FSA (First Strand Synthesis Act D Mix)
- RVT (Reverse Transcriptase)
- 1.7 ml microcentrifuge tube, RNase-free
- Microseal 'B' adhesive film

Preparation

1. Prepare the following consumables:
   - FSA—Invert to mix, and then centrifuge briefly.
   - RVT—Invert to mix, and then centrifuge briefly.
2. Save the following FSS program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 25 µl
   - 25°C for 10 minutes
   - 42°C for 15 minutes
   - 70°C for 15 minutes
   - Hold at 4°C

   Total program time is ~43 minutes.

Procedure

1. In a 1.7 ml tube on ice, combine the following volumes to prepare First Strand Synthesis (FSS) Master Mix. Multiply each volume by the number of samples.
   - FSA (9 µl)
   - RVT (1 µl)

   Reagent overage is included in the volume.
2. Pipette First Strand Synthesis Master Mix to mix.
3. Remove the seal from the PCR plate.
4. Add 8 µl First Strand Synthesis Master Mix to each well.

5. Pipette 10 times, and then seal.
6. Centrifuge at 280 × g for 10 seconds.
7. Place on the preprogrammed thermal cycler and run the FSS program.
   Each well contains a volume of 25 µl.

Synthesize Second Strand cDNA

This step removes the RNA template and synthesizes a replacement strand to generate blunt-ended, double-stranded cDNA fragments. Magnetic beads then separate the cDNA from the Second Strand Synthesis Master Mix.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- SMM (Second Strand Marking Master Mix)
- 96-well PCR plate, semiskirted
- Freshly prepared 80% ethanol (EtOH)
- Microseal 'B' adhesive film

About Reagents

- IPB
  - Use at room temperature.
  - Resuspend before each use.

Preparation

1. Prepare the following consumables:
   - RSB—Vortex and invert to mix.
   - SMM—Invert to mix, and then centrifuge briefly.
2. Prepare 80% EtOH from absolute EtOH.
3. Save the following SSS program on the thermal cycler:
   - Choose the preheat lid option and set to 40°C
   - Set the reaction volume to 50 µl
   - 16°C for 1 hour
   - Hold at 4°C

   Total program time is ~1 hour.

Procedure

Generate cDNA

1. Centrifuge the PCR plate at 280 × g for 10 seconds.
2. Invert SMM to mix, and then centrifuge briefly.
3. Add 25 µl SMM to each well.
4. Pipette 10 times, and then seal.
5. Centrifuge at 280 × g for 10 seconds.
6. Place on the preprogrammed thermal cycler and run the SSS program.
   Each well contains a volume of 50 µl.

Clean Up cDNA

1. Centrifuge the PCR plate at 280 × g for 10 seconds.
2. Resuspend IPB as follows.
   [Tube]
   - Vortex and invert for 1 minute to mix.

   [Bottle]
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.
   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
3. Add 90 µl IPB to each well.
4. Seal and shake at 2200 rpm for 1 minute.
5. Incubate at room temperature for 5 minutes.
6. Centrifuge at 280 × g for 10 seconds, and then unseal.
7. Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
8. Remove and discard all supernatant.
9. Wash beads as follows.
   a. Keep on the magnetic stand and add 200 µl fresh 80% EtOH to each well.
   b. Wait 30 seconds.
   c. Remove and discard all supernatant from each well.
10. Wash beads a **second** time.
11. Wash beads a **third** time.
12. With a 20 µl pipette, remove all residual EtOH.
13. Air-dry on the magnetic stand for 2 minutes. Do not overdry the beads.

Do not overdry the beads, as this can result in lower target recovery. Overdried beads appear light brown and cracked. If the beads overdry, immediately add RSB.

14. Remove from the magnetic stand.
15. Add 19.5 µl RSB to each well.
16. Seal and shake at 2700 rpm for 1 minute.
17. Incubate at room temperature for 2 minutes.
18. Centrifuge at 280 × g for 10 seconds, and then unseal.
19. Place on the magnetic stand and wait until the liquid is clear (~4 minutes).
20. Transfer 17.5 µl supernatant from each well to a new PCR plate.
    Small amounts of bead carryover do not affect performance.

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

This preparation is required to perform the protocol steps leading up to the next stopping point.

1. Remove DNA from storage.
2. Remove the reagents from the box and prepare as follows.

Table 3 Room Temperature Storage

Reagent	Box Name	Instructions
ST2	Illumina DNA/RNA Prep Tagmentation Buffers	Use at room temperature.
TWB	Illumina DNA/RNA Prep Tagmentation Buffers	Use at room temperature.

Table 4 2°C to 8°C Storage

Reagent	Box Name	Instructions
EBLTL	Illumina DNA/RNA Prep Tagmentation Beads	Bring to room temperature.
RSB	Illumina DNA/RNA Prep Tagmentation Beads	Bring to room temperature.

Table 5 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EPM	Illumina DNA/RNA Prep Tagmentation PCR Reagents	Thaw on ice.

Reagent	Box Name	Instructions
Index adapter plate	Illumina DNA/RNA UD Indexes	Thaw at room temperature.
TB1	Illumina DNA/RNA Prep Tagmentation PCR Reagents	Bring to room temperature.

Tagment cDNA

This step uses Enrichment Bead-Linked Transposomes to tagment double-stranded cDNA. The tagmentation process fragments cDNA and adds adapter sequences.

After tagmentation, the fragments are purified and amplified to add index adapter sequences for dual indexing and P7 and P5 sequences for clustering. For help selecting index adapters, refer to *Pooling Preparation* on page 10.

Consumables

- EBLTL (Enrichment Bead-Linked Transposomes)
- EPM (Enhanced PCR Mix)
- ST2 (Stop Tagment Buffer 2)
- TB1 (Tagmentation Buffer 1)
- TWB (Tagmentation Wash Buffer)
- 1.7 ml microcentrifuge tube
- Index adapter plate (UPDP0XXX)
- Microseal 'B' adhesive film
- Nuclease-free ultrapure water

About Reagents

- UDP0XXX—Each well of the index adapter plate is single-use and contains > 10 µl UDP0XXX, which are premixed Index 1 (i7) and Index 2 (i5) adapters.
- The row and column labels are printed on the underside of the index adapter plate. Raise the plate overhead to check the labels.

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, refer to the SDS at support.illumina.com/sds.html.

Preparation

1. Prepare the following consumables:
   - EBLTL—Vortex to mix until beads are resuspended.
   - EPM—Invert to mix, and then centrifuge briefly.
   - Index adapter plate—Vortex to mix, and then centrifuge at 1000 × g for 1 minute.
   - ST2—Vortex to mix, and then centrifuge briefly.
   - TB1—Vortex to mix.
   - TWB—Vortex to mix.
2. If the ST2 tube has precipitate, proceed as follows.
   a. Heat at 37°C for 10 minutes.
   b. Vortex until precipitate is dissolved.
   c. Return to room temperature.
3. Save the following TAG program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 55°C for 5 minutes
   - Hold at 10°C

   Total program time is ~5 minutes.
4. Save the following 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
   - X 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

Total program time is ~50–60 minutes.

Input	Number of Cycles (X)*
High-quality RNA with DV200 > 80%	14
FFPE and RNA with DV200 < 80%	17

* To achieve the desired library yield and specificity, optimize the number of PCR cycles for your sample type and input.

Procedure

Tagment With EBLTL

1. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. In a 1.7 ml tube, combine the following volumes to prepare Tagmentation Master Mix. Multiply each volume by the number of samples.
   - TB1 (11.5 µl)
   - EBLTL (11.5 µl)
   - Nuclease-free ultrapure water (14.5 µl)

   Reagent overage is included in the volume.
3. Vortex the Tagmentation Master Mix to resuspend.
4. Add 32.5 µl Tagmentation Master Mix to each well.
5. Pipette to mix, and then seal.
6. Place on the preprogrammed thermal cycler and run the TAG program.
   Each well contains a volume of 50 µl.

Wash Tagmented cDNA

1. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. Incubate at room temperature for 2 minutes.
3. Add 10 µl ST2 to each well.
4. Seal and shake at 2200 rpm for 1 minute.
5. Incubate at room temperature for 5 minutes.
6. Centrifuge at 280 × g for 10 seconds, and then unseal.
7. Place on the magnetic stand and wait until the liquid is clear (~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 2000 rpm for 1 minute.
d. Centrifuge at 280 × g for 3 seconds.
e. Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
f. Remove and discard all supernatant.

10. Wash beads a **second** time.
11. Wash beads a **third** time. Do not discard supernatant.
    TWB remains in the wells to prevent over-drying.
12. Keep on the magnetic stand.
    Each well contains 100 µl beads with tagmented cDNA.

Amplify Tagmented DNA

1. Combine the following volumes to prepare PCR Master Mix. Multiply each volume by the number of samples, and add the total calculated volumes to the 1.7 ml tube.
   - EPM (23 µl)
   - Nuclease-free ultrapure water (23 µl)

   Reagent overage is included in the volume.
2. Vortex PCR Master Mix to mix.
3. Keeping the plate on the magnetic stand, remove and discard all TWB supernatant.
4. With a 20 µl pipette, remove all residual TWB.
   Foam is normal and does not affect the library.
5. Remove from the magnetic stand.
6. Add 40 µl PCR Master Mix to each well.
7. Using a new pipette tip for each well, pierce the foil covering the index adapter plate wells that you intend to use.
8. Add 10 µl UDP0XXX to each well. Transfer volumes from the index adapter plate to the PCR plate.
9. Seal and shake at 2000 rpm for 1 minute.
10. Centrifuge at 280 × g for 3 seconds.
11. Place on the preprogrammed thermal cycler and run the TAG_PCR program.
    Each well contains 50 µl beads with DNA attached.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days. Alternatively, leave on the thermal cycler for up to 24 hours.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next stopping point.

1. Remove sample from storage.
2. Remove the reagents from the box and prepare as follows.

Table 6 Room Temperature Storage

Reagent	Box Name	Instructions
IPB	Illumina DNA/RNA Prep-Tagmentation Buffers	Use at room temperature.

Table 7 2°C to 8°C Storage

Reagent	Box Name	Instructions
RSB	Illumina DNA/RNA Prep-Tagmentation (S) Beads	Bring to room temperature.

Clean Up Library

This step uses magnetic beads to purify the tagmented library.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- EtOH (Freshly prepared 80% ethanol)
- 96-well PCR plate, semiskirted
- Microseal 'B' adhesive film

About Reagents

- IPB
  - Use at room temperature.
  - Resuspend before each use.

Preparation

1. Prepare the following consumables:
   - RSB—Vortex and invert to mix.
   - EtOH—Prepare 80% EtOH from absolute EtOH.

Procedure

1. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
3. Transfer 45 µl supernatant from each well to a new PCR plate.
4. Resuspend IPB as follows.

   [Tube]

   - Vortex and invert for 1 minute to mix.

   [Bottle]

   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.

   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
5. Add 81 µl IPB to each well containing a sample.
6. Seal and shake at 2200 rpm for 1 minute.
7. Incubate at room temperature for 5 minutes.
8. Centrifuge at 280 × g for 10 seconds, and then unseal.
9. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
10. Remove and discard all supernatant.
11. Wash beads as follows.

    a. Keep on the magnetic stand and add 200 µl fresh 80% EtOH to each well.

    b. Wait 30 seconds.

    c. Remove and discard all supernatant from each well.
12. Wash beads a **second** time.
13. Wash beads a **third** time.
14. With a 20 µl pipette, remove all residual EtOH.
15. Air-dry on the magnetic stand for 2 minutes. Do not overdry the beads.

    Do not overdry the beads, as this can result in lower target recovery. Overdried beads appear light brown and cracked. If the beads overdry, immediately add RSB.
16. Remove from the magnetic stand.
17. Add 17 µl RSB to each well.
18. Seal and shake at 2700 rpm for 1 minute.
19. Incubate at room temperature for 2 minutes.
20. Centrifuge at 280 × g for 10 seconds, and then unseal.
21. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

22. Transfer 15 µl supernatant from each well to a new PCR plate.

SAFE STOPPING POINT

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

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next stopping point.

1. Remove DNA from storage.
2. Remove the reagents from the box and prepare as follows.

Table 8 2°C to 8°C Storage

Reagent	Box Name	Instructions
EHB2	Illumina RNA Fast Hyb Enrichment Beads + Buffers	Use at room temperature.
ET2	Illumina RNA Fast Hyb Enrichment Beads + Buffers	Use at room temperature.
RSB	Illumina RNA Fast Hyb Enrichment Beads + Buffers	Use at room temperature.
SMB4	Illumina RNA Fast Hyb Enrichment Beads + Buffers	Bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends, and vortex to resuspend.

Table 9 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EE1	Illumina RNA Fast Hyb Enrichment PCR + Buffers	Thaw at room temperature.
EEW	Illumina RNA Fast Hyb Enrichment PCR + Buffers	Thaw at room temperature.
HP3	Illumina RNA Fast Hyb Enrichment PCR + Buffers	Thaw at room temperature.
NHB2	Illumina RNA Fast Hyb Enrichment PCR + Buffers	Thaw and use at room temperature.

3. Remove an enrichment probe panels from the box and prepare as follows.

Table 10 Enrichment Probe Panels -25°C to -15°C Storage

Panel name	Reagent	Instructions
Illumina Exome Panel	CEX	Thaw at room temperature.
Pan-Coronavirus Panel	Pan-CoV	Thaw at room temperature.
Illumina Custom Enrichment Panel v2	ICEPv2	Thaw at room temperature.
Respiratory Virus Oligo Panel v2 (Respiratory Virus Enrichment Kit (RVEK) uses RVOPv2 panel)	RVOPv2	Thaw at room temperature.
Respiratory Pathogen ID/AMR Panel	RPIP	Thaw at room temperature.

Normalize Library

This step quantifies and normalizes libraries, then combines them into one pool for one- or three-plex enrichment. Results are optimized for 200 ng of each library.

Due to the highly complex nature of infectious disease and microbiology samples, which can contain both host and pathogen nucleic acid, using normalized inputs by equal mass is not recommended. Instead use an equal volume of extracted nucleic acid per sample without normalization. This strategy applies to the Respiratory Virus Oligo Panel, Respiratory Pathogen ID/AMR Panel, Viral Surveillance Panel, and Pan Coronavirus Panel. Use the maximum volume of libraries for best results.

Consumables

- RSB (Resuspension Buffer)
- 96-well PCR plate, semiskirted
- Qubit dsDNA BR Assay Kit
- **[Optional]**
  - Agilent DNA 1000 Kit
  - D1000 ScreenTape

Preparation

1. Prepare the following consumables:
   - RSB (refrigerated)—Vortex and invert to mix.

Procedure

1. Analyze 1 µl library with the Qubit dsDNA BR Assay Kit.
2. **[Optional]** Analyze 1 µl library with the one of the following analysis systems:
   - Agilent 2100 Bioanalyzer System and DNA 1000 Kit.
   - Agilent TapeStation System and D1000 ScreenTape.
3. **[All Other Libraries]** Dilute libraries in RSB as follows.
   - For one-plex enrichment, dilute one 200 ng library to a volume of 7.5 µl.
   - For three-plex enrichment, dilute three 200 ng libraries to a volume of 2.5 µl each.
4. **[Diluted Libraries]** In one well of a new PCR plate, combine the applicable number of 200 ng libraries as follows.

Number of Libraries (Enrichment Plexity)	Total Mass (ng)	Total Volume (µl)
1	200	7.5
3	600	7.5

   If the total volume is > 7.5 µl, use a vacuum concentrator to concentrate the pooled sample to 7.5 µl. If you are using an Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa), take double the required mass, concentrate to 15 µl and use 7.5 µl for the next step. For example, if the desired final mass is 200 ng, use 400 ng total and concentrate to the minimum final volume of 15 µl. Use 7.5 µl this final volume for the next protocol step.
   - If you are using a vacuum concentrator, use a no heat setting and a medium drying rate.
   - If you are using an Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa), you do not need to rinse the device before use.
     - Most of the volume filters through in 5 minutes.
     - Larger starting volumes can take up to 30 minutes to filter.

Hybridize Probes

This step adds capture probes to pooled libraries to target regions of interest. The procedure uses enrichment reagents and oligos from an enrichment panel.

Consumables

- EHB2 (Enrich Hyb Buffer 2)
- NHB2 (Hyb Buffer 2+IDT NXT Blockers)
- Enrichment panel
  - **[Illumina Custom Enrichment Panel v2 (ICEPv2)]**

    - Nucleus-free water, 1.5 µl
    - Microcentrifuge tubes, 1.5 ml
- 96-well PCR plate, semiskirted
- Microseal 'B' adhesive film

About Reagents

- NHB2
  - Precipitates and separates during storage.
  - Follow the preparation instructions before first use.

Preparation

1. Preheat the microheating system to 50°C.
2. Prepare the following consumables:
    - EHB2—Vortex to mix. If crystals and cloudiness are observed, repeat vortex, or pipette up and down to mix well until the solution is clear.
    - Enrichment panel—Thaw at room temperature, and then vortex to mix.
        - **[ICEPv2]**—In a 1.5 ml tube, combine 1 µl ICEPv2 and 1.5 µl nucleus-free water.
    - NHB2—Vortex to mix. Lay in the preheated microheating system and incubate for 5 minutes.
    - SMB4—If you are proceeding to the next procedure immediately after the 90 minute hold in the HYB program, bring to room temperature. If you are extending the hold time, bring to room temperature at least 30 minutes before the HYB program ends.
3. Mix preheated NHB2 as follows.
    a. Vortex three times for 10 seconds each.
    b. Pipette to resuspend fully. Keep warm until use to prevent the reformation of precipitates.
4. If EHB2 or NHB2 appears crystallized or cloudy, vortex or pipette until clear.
5. Save the following HYB program on the thermal cycler:
    - Choose the preheat lid option and set to 100°C
    - Set the reaction volume to 25 µl
    - 95°C for 5 minutes
    - 18 cycles of 1 minute each:
        - 94°C for the first cycle
        - Decrease 2°C per subsequent cycle
    - 58°C for 90 minutes

- Hold at 58°C for ≤ 24 hours

The minimum hybridization time at 58°C is 90 minutes. Extending the hold to up to 24 hours is optional for overnight hybridization. Without the extended hold, total program time is ~2 hours.

Procedure

1. Add the following volumes to each well of a new PCR plate ***in the order listed***.
   a. 200 ng library or 600 ng three-plex pre-enriched library pool (7.5 µl)
   b. NHB2 (12.5 µl)
   c. Enrichment panel (2.5 µl)
      - **[Diluted ICEPv2]**
   d. EHB2 (2.5 µl)
2. Pipette 10 times to mix, and then seal.
3. Centrifuge at 280 × g for 3 seconds.
   EHB2 can make the reaction appear cloudy, which is normal.
4. Place on the preprogrammed thermal cycler and run the HYB program.
5. Allow to incubate at 58°C for 90 minutes to 24 hours.
   Each well contains a volume of 25 µl.

If the reaction falls below room temperature precipitates can form, impacting the run results.

Capture Hybridized Probes

This step uses magnetic beads to capture probes hybridized to the targeted library fragments of interest. Heated washes remove nonspecific binding from the beads. The enriched library is then eluted from the beads.

Consumables

- EE1 (Enrichment Elution Buffer 1)
- EEW (Enhanced Enrichment Wash)
- ET2 (Elute Target Buffer 2)
- HP3 (2 N NaOH)
- SMB4 (Streptavidin Magnetic Beads 4)
- 1.7 ml microcentrifuge tube
- 96-well PCR plate, semiskirted (2)
- Microseal 'B' adhesive film

Preparation

1. Preheat the microheating system to 58°C.
2. Prepare the following consumables:
   - EE1—Pipette to mix.
   - EEW—Vortex to mix.
   - ET2—Vortex to mix.
   - HP3—Vortex to mix.
   - SMB4
     - Bring to room temperature before use.
     - Vortex to mix until beads are resuspend. If precipitate or the bead pellet is present, make sure the reagent reaches room temperature, pipette up and down to release the pellet, and then vortex to resuspend.
3. Place EEW in the preheated microheating system, and keep heated for subsequent steps.
4. Set the thermal cycler as follows.
   - If it has an incubation option, set it to 58°C.
   - If it does not have an incubation option, save the following Incubation program:
     - Choose the preheat lid option and set to 70°C
     - Set the reaction volume to 100 µl
     - Hold at 58°C

Procedure

Capture

1. Centrifuge the sample plate or tube at 280 × g for 10 seconds.
2. Vortex SMB4 to resuspend. If precipitate or the bead pellet is present, make sure the reagent reaches room temperature, pipette up and down to release the pellet, and then vortex to resuspend.
3. Add 62.5 µl to each well or tube.
4. Slowly pipette until the beads are resuspended, and then seal.
5. Place the sample plate or tube in the preheated thermal cycler, close the lid, and incubate for 15 minutes.
   The thermal cycler runs continuously through the capture and four washes.
6. Centrifuge the sample plate or tube at 280 × g for 10 seconds.
7. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
8. Remove and discard all supernatant from each well or tube.

Wash

1. Remove from the magnetic stand.
2. Add 50 µl preheated EEW to each well or tube. To mix, use one of the following options:
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each. Do not centrifuge.
   - **[Plate]** Seal and shake at 2400 rpm for 4 minutes.
3. Return unused EEW to the microheating system and keep heated.
4. Place the sample plate or tube on the thermal cycler and incubate for 5 minutes at 58°C.
5. Centrifuge at 280 × g for 3 seconds.
6. Place the plate or microcentrifuge tube on a magnetic stand and wait until the liquid is clear (~2 minutes).
7. Using a pipette set to 200 µl, remove and discard all supernatant from each well or tube.

Second and Third Wash

1. Remove from the magnetic stand.
2. Add 50 µl preheated EEW to each well or tube. To mix, use one of the following options:
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each. Do not centrifuge.
   - **[Plate]** Seal and shake at 2000 rpm for 1 minute.
3. Return unused EEW to the microheating system and keep heated.
4. Place the sample plate or tube on the thermal cycler and incubate for 5 minutes at 58°C.
5. Centrifuge at 280 × g for 3 seconds.
6. Place the plate or microcentrifuge tube on a magnetic stand and wait until the liquid is clear (~2 minutes).
7. Using a pipette set to 200 µl, remove and discard all supernatant from each well or tube.
8. Repeat steps 1–7 for a **third** wash.

Transfer Wash

1. Remove the plate or tube from the magnetic stand.
2. Add 50 µl preheated EEW to each well or tube. Mix thoroughly as follows.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
   - **[Plate]** Seal and shake at 2400 rpm for 1 minute.
3. Seal and centrifuge at 280 × g for 3 seconds.
4. Transfer samples to a new MIDI plate or new tube strip.

   Residual reagents can carryover from previous plates or tubes, inhibiting downstream PCR. Transferring the reagents to a new vessel minimizes that error.

5. Seal and centrifuge at 280 × g for 3 seconds.
6. Place the sample plate or tube on the thermal cycler and incubate for 5 minutes at 58°C.
7. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
8. Remove and discard all supernatant from each well or tube.
9. Use a 20 µl pipette to remove and discard residual liquid from each well or from the tube.

Elute

1. Combine the following volumes to prepare an Elution Master Mix. Multiply each volume by the number of samples being processed.
   Additional reagent is included in the volume to ensure accurate pipetting due to the potential of reagent foaming.
   - EE1 (28.5 µl)
   - HP3 (1.5 µl)
2. Pipette the Elution Master Mix to mix, and then set aside at room temperature.
3. Remove the sample plate or tube from the magnetic stand.
4. Add 23 µl Elution Master Mix to each well or to the tube, and then use one of the following options to mix:
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
   - **[Plate]** Seal plate and shake at 2600 rpm for 1 minute.
5. Incubate the plate or tube at room temperature for 2 minutes.
6. Centrifuge at 280 × g for 30 seconds.
7. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
8. Transfer 21 µl supernatant from the MIDI plate or tube strip to the corresponding well of a new 96-well PCR plate or a new 8-tube strip.
9. Add 4 µl ET2 to each well or to the tube containing 21 µl supernatant.
10. Set pipette to 20 µl and slowly pipette each well or the tube 10 times to mix.
11. Centrifuge the sample plate or the tube at 280 × g for 30 seconds.

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

This preparation is required to perform the protocol steps leading up to the next stopping point.

1. Remove DNA from storage.
2. Remove the reagents from the box and prepare as follows.

Table 11 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EPM	Illumina RNA Fast Hyb Enrichment PCR+Buffers	Thaw on ice.
PPC	Illumina RNA Fast Hyb Enrichment PCR+Buffers	Thaw on ice.

Amplify Enriched Library

This step uses a 14-cycle PCR program to amplify the enriched library.

Consumables

- EPM (Enhanced PCR Mix)
- PPC (PCR Primer Cocktail)
- Microseal 'B' adhesive film

Preparation

1. Prepare the following consumables:
   - EPM—Invert to mix, and then centrifuge briefly.
   - PPC—Invert to mix, and then centrifuge briefly.
2. Save the following AMP program on the thermal cycler.
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 98°C for 30 seconds
   - 14 cycles of:
     - 98°C for 10 seconds
     - 60°C for 30 seconds
     - 72°C for 30 seconds
   - 72°C for 5 minutes
   - Hold at 10°C
   - Total program time is ~35 minutes.

Procedure

1. Centrifuge the sealed plate at 280 × g for 10 seconds.
2. Add 5 µl PPC to each well of the PCR plate.
3. Add 20 µl EPM to each well.

4. Seal and shake at 2000 rpm for 1 minute.
5. Centrifuge at 280 × g for 10 seconds.
6. Place on the preprogrammed thermal cycler and run the AMP program.
   Each well contains a volume of 50 µl.

SAFE STOPPING POINT

If you are stopping, store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler for up to 24 hours.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next stopping point.

1. Remove DNA from storage.
2. Remove the reagents from the box and prepare as follows.

Table 12 Room Temperature Storage

Reagent	Box Name	Instructions
IPB	Illumina DNA/RNA Prep-Tagmentation Buffers	Use at room temperature.

Table 13 2°C to 8°C Storage

Reagent	Box Name	Instructions
RSB	Illumina RNA Fast Hyb Enrichment Beads+Buffers	Bring to room temperature.

Clean Up Enriched Library

This step uses magnetic beads to purify the enriched library.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- 96-well PCR plate, semiskirted
- Freshly prepared 80% ethanol (EtOH)
- Microseal 'B' adhesive film

About Reagents

- IPB
  - Use at room temperature.
  - Resuspend before each use.

Preparation

Prepare the following consumables:

- RSB—Vortex and invert to mix.
- EtOH—Prepare 80% EtOH from absolute EtOH.

Procedure

1. Centrifuge the sealed plate at 280 × g for 10 seconds.
2. Resuspend IPB as follows.
   [Tube]
   - Vortex and invert for 1 minute to mix.

   [Bottle]
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.
   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
3. Add 90 µl IPB to each well.
4. Seal and shake at 2200 rpm for 1 minute.
5. Incubate at room temperature for 5 minutes.
6. Centrifuge at 280 × g for 10 seconds, and then unseal.
7. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
8. Remove and discard all supernatant.
9. Wash beads as follows.
   a. Keep on the magnetic stand and add 200 µl fresh 80% EtOH to each well.
   b. Wait 30 seconds.
   c. Remove and discard all supernatant from each well.
10. Wash beads a **second** time.
11. Wash beads a **third** time.
12. With a 20 µl pipette, remove all residual EtOH.
13. Air-dry on the magnetic stand for 2 minutes. Do not overdry the beads.

Do not overdry the beads, as this can result in lower target recovery. Overdried beads appear light brown and cracked. If the beads overdry, immediately add RSB.

14. Remove from the magnetic stand.
15. Add 32 µl RSB to each well.
16. Seal and shake at 2600 rpm for 1 minute.
17. Incubate at room temperature for 2 minutes.
18. Centrifuge at 280 × g for 10 seconds, and then unseal.
19. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
20. Transfer 30 µl supernatant from each well to a new PCR plate.

SAFE STOPPING POINT

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

Check Enriched Library

Check the enriched library using both of the following methods:

- To quantify library concentration (ng/µl), analyze 1 µl enriched library with the Qubit dsDNA HS Assay kit.
- To qualify the library, analyze 1 µl enriched library with one of the following analysis systems:
  - Agilent 2100 Bioanalyzer System and DNA 1000 Kit.
  - Agilent TapeStation System and D1000 ScreenTape.
  - Agilent TapeStation System and High Sensitivity D1000 ScreenTape

Typical libraries show a broad size distribution of 250–1000 bp, as shown in the following examples.

Figure 1 Example Bioanalyzer Trace

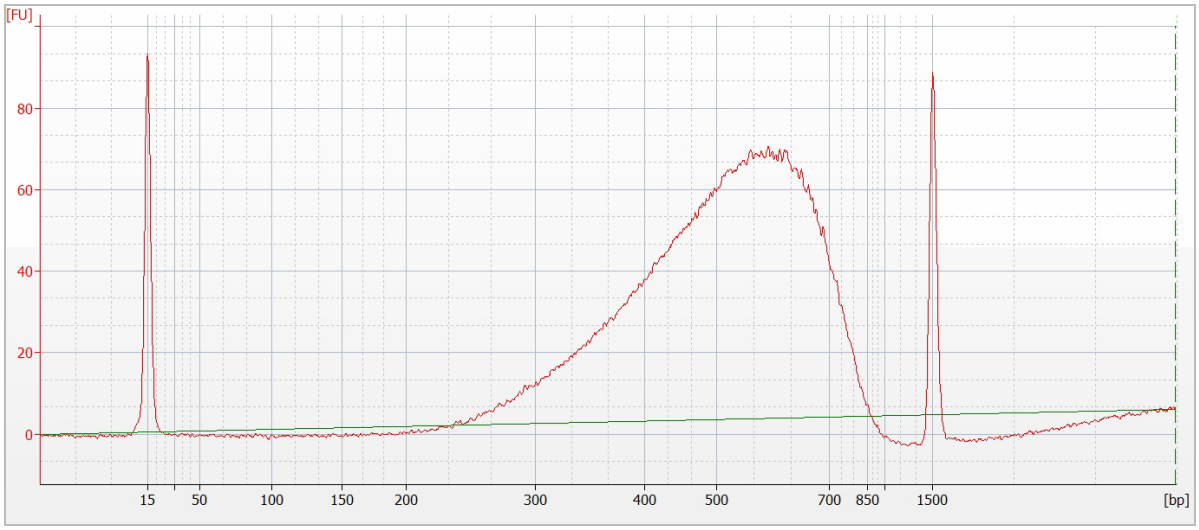
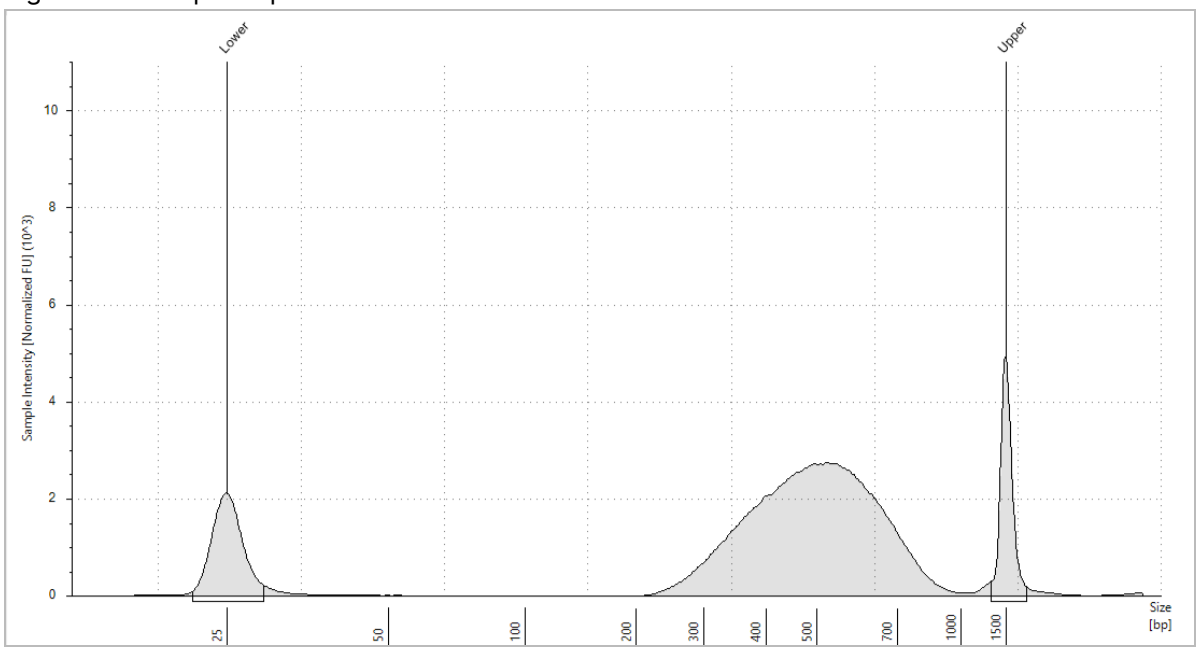


Figure 2 Example TapeStation Trace



Dilute Library to the Starting Concentration

This step dilutes libraries to the starting concentration for the NovaSeq 6000, NextSeq 500, NextSeq 550, or System System. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration. Illumina recommends paired-end runs for sequencing. The number of cycles per Index Read is 10, and the number of cycles per read varies depending on the sequencing system.

1. Obtain the molarity value of the library or pooled libraries using the applicable method:
   - For libraries quantified with either a Bioanalyzer or TapeStation system only, use the molarity value obtained for the library.
   - For libraries quantified with a Bioanalyzer or TapeStation system and a Qubit assay, use the following formula to calculate the molarity value. Apply the average size from the Bioanalyzer or TapeStation system results and the concentration from the Qubit assay results.

$$\frac{\text{concentration in ng}/\mu\text{l}}{660\ \text{g/mol} \times \text{average library size in bp}} \times 10^6 = \text{Molarity (nM)}$$

2. Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system.

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
MiniSeq	2	2
MiSeq	4	10

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
MiSeq i100*	0.8	80
NextSeq 550 and NextSeq 500	2	0.8
NovaSeq 6000	0.6	120
NovaSeq X	2	175

* Denaturation performed onboard. Refer to the system guide.

3. Dilute each library to the starting concentration for your system using RSB. Combine 10 µl each diluted library in a tube to pool libraries.
4. Follow the denature and dilute instructions for your system to dilute libraries to the final loading concentration.

Resources and References

The Illumina RNA Prep with Enrichment, (L) Tagmentation support pages on the Illumina Support Center provide additional resources. These resources include training, compatible products, and other considerations. Always check support pages for the latest versions.

Resource	Description
Index Adapters Pooling Guide	Provides recommendations to plan indexing and pooling strategies.
Illumina Adapter Sequences	Provides adapter sequences for Illumina library prep kits.

Revision History

Document	Date	Description of Change
Document # 1000000124435 v06	June 2026	Added Illumina Custom Enrichment Panel v2. Standardized thermal cycler specifications.
Document # 1000000124435 v05	October 2025	Updated SMB3 to SMB4. Updated SMB4 thaw and preparation instructions. Updated thermal cyclers. Added MiSeq i100 and NovaSeq X.

Document	Date	Description of Change
Document # 1000000124435 v04	February 2025	Added values for NovaSeq X and MiSeq i100. Added plexity and enrichment reaction information for the reagent kit options. Added Illumina DNA/RNA UD index adapter kit and Pan-Coronavirus Panel specifications. Updated the specifications for the following consumables and equipment: • Bioanalyzer and TapeStation systems and kits • Thermal cycler Corrected the starting concentration for the NextSeq 550 and NextSeq 500 instruments Replaced references to Agencourt AMPure XP with Illumina Purification Beads (IPB). Added TapeStation analysis instructions and trace graphic. Updated the Bioanalyzer trace graphic. Updated the additional resources documentation references. Updated as follows, to align with Illumina documentation guidelines. Converted to HTML format. Restructured the product contents tables. Removed catalog numbers for product subcomponents. Collated the reagent thaw instructions into protocol preparation sections. Removed the acronyms list. Acronyms are now written in full on first mention.
Document # 1000000124435 v03	April 2021	Updated SMB to SMB3.
Document # 1000000124435 v02	October 2020	Added Respiratory Pathogen ID/AMR panel information and Target Enrichment Kit catalog numbers.
Document # 1000000124435 v01	August 2020	Added Respiratory Virus Oligos Panel v2. Added dilution and pooling instructions in the normalization procedure for 3-plex Respiratory Virus Panel libraries Updated workflow diagram description to include the number of samples used to calculate processing times. Corrected formatting of index kit names.
Document # 1000000124435 v00	June 2020	Initial release.

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