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Illumina Rapid Viral Surveillance Panel v2

Product Documentation

ILLUMINA PROPRIETARY
Document #200076914 v00
June 2026

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Overview

The Illumina Rapid Viral Surveillance Panel v2 kit integrates the RNAseq library preparation with hybrid capture probes to enrich the probe targets. The library prep method combines cDNA synthesis and tagmentation into a single step to significantly reduce assay time. Dual indexes are added during PCR and the resulting libraries are normalized for three-plex enrichment and further amplification.

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 (EBLTS) to create larger inserts
- Unique dual (UD) indexing with Illumina DNA/RNA UD Indexes
- High-quality sequencing data from a range of input, 1–1000 ng total RNA
- cDNA synthesis and tagmentation in a single step

Input Recommendations

The protocol is optimized for the following input sample types:

- 1–1000 ng of purified total RNA, DNA, or total nucleic acid.
- 20–1000 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.

Due to the highly complex nature of real world samples, which can contain both host and pathogen nucleic acid, using normalized inputs by equal mass is not recommended. Instead we recommend using equal volume of extracted nucleic acid per sample without normalization.

For infectious disease and microbiology panels:

- 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 DNA and RNA).
- 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 DNA and RNA) is recommended.

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

Completing the Illumina Rapid Viral Surveillance Panel v2 protocol requires the Illumina Rapid Viral Surveillance Panel v2 and index adapters. The number of index adapters required depends on the number of samples to be uniquely indexed for your experiment.

- The required library preparation kits provide reagents for denaturing, cDNA synthesis, library prep, and enrichment in a 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.

Table 1 Library Prep Reagents

Catalog Item	Catalog #	Box Name	Box #
Illumina Rapid Viral Surveillance Panel v2 (96 samples)	20158826	Illumina Rapid RNA Prep	20115932
		Illumina DNA/RNA Prep Tag Buffers 96 v2	20150313
		Illumina DNA/RNA Prep-Tagmentation (S) Beads	20026214
		IlluminaDNA/RNA Fast Hyb Enrich Beads 96	20026210
		IlluminaRNA Single Hyb Enrich PCR 16	20041737
		Viral Surveillance Panel v2	20107489

Illumina Rapid Viral Surveillance Panel v2 Kit Contents

Table 2 Illumina Rapid RNA Prep, Store at -25°C to -15°C

Quantity	Reagent	Description
4	EPH3	Elute, Prime, Fragment High Concentration Mix
2	CBM	cDNA Buffer Mix
1	CEM	cDNA Enzyme Mix
1	LRA	Library Reducing Agent
4	EPM	Enhanced PCR Mix

Table 3 Illumina DNA/RNA Prep Tag Buffers 96 v2, Store at Room Temperature*

Quantity	Reagent	Description
4	ST2	Stop Tagment Buffer 2
1	TWB2	Tagmentation Wash Buffer
2	IPB	Illumina Purification Beads

*Shipped at 2°C to 8°C.

Table 4 Illumina DNA/RNA Prep-Tagmentation (S) Beads, Store at 2°C to 8°C

Quantity	Reagent	Description
4	EBLTS	Enrichment Bead-Linked Transposomes
2	RSB	Resuspension Buffer

Table 5 Illumina RNA Single Hyb Enrich PCR 96, Store at -25°C to -15°C*

Quantity	Reagent	Description
2	EE1	Enrichment Elution Buffer 1
4	EEW	Enhanced Enrichment Wash
2	EPM	Enhanced PCR Mix
1	HP3	2 N NaOH
1	NHB2	Hyb Buffer 2+IDT NXT Blockers
1	PPC	PCR Primer Cocktail

*Shipped at 2°C to 8°C.

Table 6 Illumina DNA/RNA Fast Hyb Enrich Beads 96, Store at 2°C to 8°C

Quantity	Reagent	Description
1	EHB2	Enrich Hyb Buffer 2
1	ET2	Elute Target Buffer 2
1	RSB	Resuspension Buffer
2	SMB4	Streptavidin Magnetic Beads 4

Index Adapters

Index adapters are not included in the Illumina Rapid Viral Surveillance Panel v2 kit and must be purchased separately.

Table 7 Index Adapters

Name	Catalog #
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

Index Adapter Kit Contents

Each kit contains one plate of indexes and one plate of anchors.

Table 8 Illumina DNA/RNA UD Indexes, 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

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
Centrifuge tubes, conical (15 ml or 17 ml)	General lab supplier
Pipette tips, 10 µl	General lab supplier
Pipette tips, 20 µl	General lab supplier
Pipette tips, 200 µl	General lab supplier
Pipette tips, 1000 µl	General lab supplier
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
Lab tape	General lab supplier
Microseal 'B' PCR Plate Sealing Film	Bio-Rad, catalog # MSB1001
Microcentrifuge tubes, 1.5 ml	General lab supplier
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: • **[TapeStation System]** D1000 ScreenTape • **[TapeStation System]** High Sensitivity D1000 ScreenTape	One of the following suppliers: • Agilent Technologies, catalog # 5067–5582 • Agilent Technologies, catalog # 5067–5584
One of the following kits: • Qubit dsDNA BR Assay Kit • Qubit dsDNA HS Assay Kit	One of the following suppliers: • Thermo Fisher Scientific, catalog # Q32850 or Q32853 • Thermo Fisher Scientific, catalog # Q33230 or Q33231
Qubit Assay Tubes	Thermo Fisher Scientific, catalog # Q32856
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, catalog # 89094-658
Ultrapure water, nuclease-free	General lab supplier

Consumable	Supplier
[Optional] One of the following Kapa Library Quantification Kits: • Complete kit (Universal) • qPCR MasterMix (Universal) and Primer Premix only	One of the following suppliers: • Roche, catalog # 07960140001 • Roche, catalog # 07960441001

Equipment

Equipment	Supplier
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
One of the following quality analysis instruments: • 4150 TapeStation System • 4200 TapeStation System	One of the following suppliers: • Agilent Technologies, catalog # G2992AA • Agilent Technologies, catalog # G2991BA
One of the following thermal mixers: • BioShake iQ High-Speed Thermal Mixer • BioShake XP High-Speed Thermal Mixer	One of the following suppliers: • Q Instruments, catalog # 1808–0506 • Q Instruments, catalog # 1808–0505
Vortexer	General lab supplier
[Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa)	Millipore catalog # UFC503008
[Optional] qPCR instrument	General lab supplier
[Optional] Qubit 2.0 Fluorometer	Thermo Fisher Scientific, catalog # Q32866

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 Rapid Viral Surveillance Panel v2 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

It is recommended to pool samples with similar viral titer for 3-plex enrichment. Pooling samples with an above and/or below average viral titer might bias results. Single-plex enrichment is recommended for samples with low viral titer.

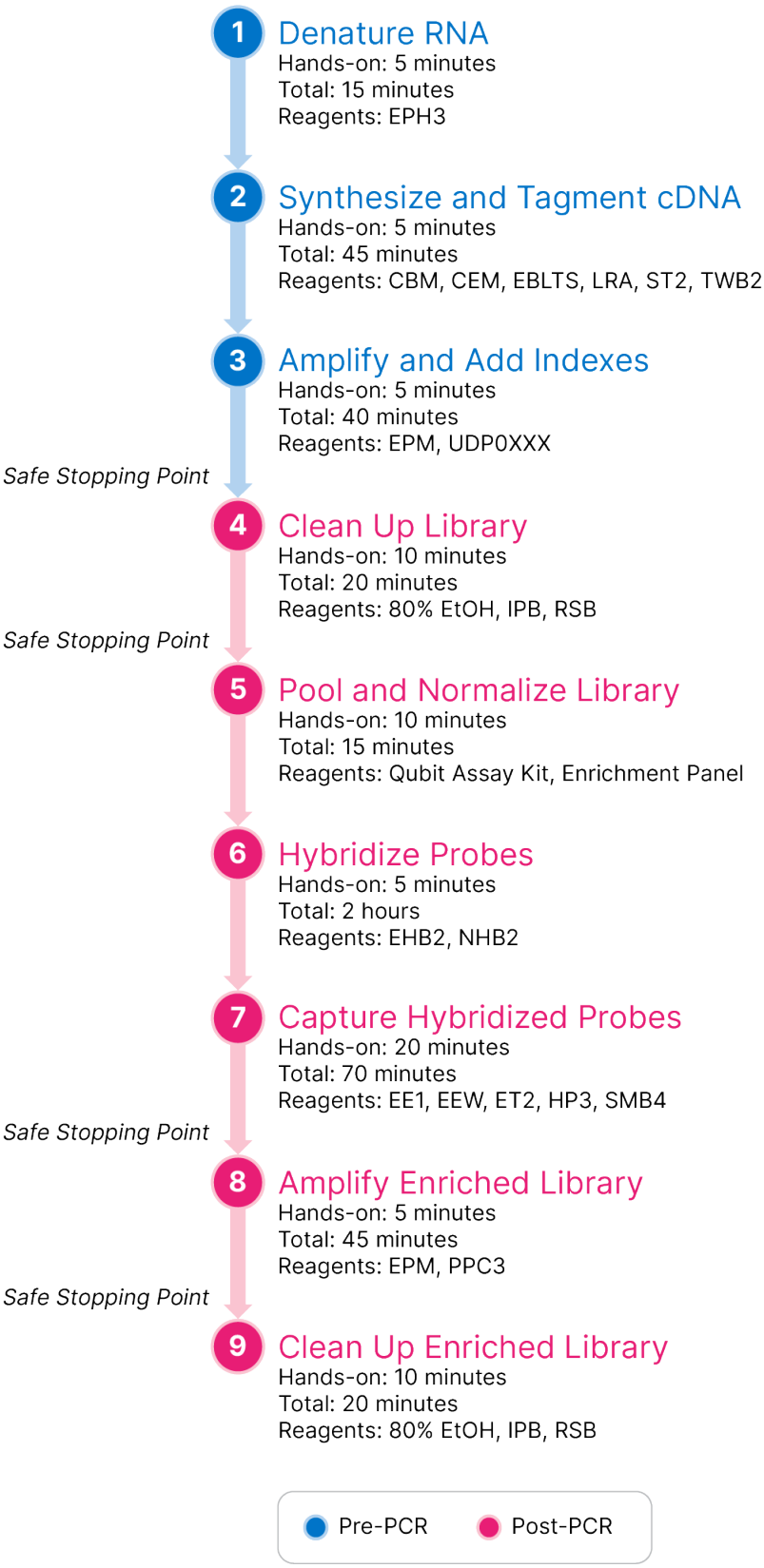
When pooling libraries, record information about your samples before starting library prep. Use a recording tool compatible with your sequencing system and libraries. For compatibility information, refer to Illumina support pages for Illumina Rapid Viral Surveillance Panel v2 or your system.

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 Rapid Viral Surveillance Panel v2 Workflow

The following diagram provides an overview of the Illumina Rapid Viral Surveillance Panel v2 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 EBLTS 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 sample from storage.
2. Remove the reagents from the box and prepare as follows.

Table 9 Room Temperature Storage

Reagent	Box Name	Instructions
ST2	Illumina DNA/RNA Prep Tag Buffers 96 v2	Use at room temperature.
TWB2	Illumina DNA/RNA Prep Tag Buffers 96 v2	Use at room temperature.

Table 10 2°C to 8°C Storage

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

Table 11 -25°C to -15°C Storage

Reagent	Box Name	Instructions
LRA	Illumina Rapid RNA Prep	Thaw at room temperature.
EPH3	Illumina Rapid RNA Prep	Thaw at room temperature.

Reagent	Box Name	Instructions
EPM	Illumina Rapid RNA Prep	Thaw at room temperature.
Index Adapters	ILMN DNA/RNA UD Index Set A, B, C or D	Thaw at room temperature.
CBM	Illumina Rapid RNA Prep	Thaw at room temperature.
CEM	Illumina Rapid RNA Prep	Thaw at room temperature.

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:
   a. Choose the preheat lid option and set to 100°C
   b. Set the reaction volume to 17 µl
   c. 65°C for 5 minutes
   d. Hold at 4°C
   Total program time is ~8 minutes.

Procedure

1. In each well of a new PCR plate, dilute 1–1000 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, and then seal.
4. Place on the preprogrammed thermal cycler and run the DEN_RNA program.

Synthesize and Tagment cDNA

This step converts the RNA fragments to complementary DNA (cDNA) and tagments the cDNA with Enrichment Bead-Linked Transposomes.

Consumables

- CBM (cDNA Buffer Mix)
- CEM (cDNA Enzyme Mix)
- EBLTS (Enrichment Bead-Linked Transposomes)
- LRA (Library Reducing Agent)
- ST2 (Stop Tagment Buffer 2)
- TWB2 (Tagmentation Wash Buffer)
- 1.7 ml microcentrifuge tube, RNase-free
- Microseal 'B' adhesive film
- Nuclease-free ultrapure water

About Reagents

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Ventilation should be appropriate for handling of hazardous materials in reagents. 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:
   - CBM—Vortex to mix. Centrifuge briefly.
   - CEM—Flick to mix, and then centrifuge briefly.
   - EBLTS—Vortex to mix until beads are resuspended.
   - LRA—Vortex and invert to mix. Centrifuge briefly.
   - ST2—Vortex to mix.
   - TWB2—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 RLP program on the thermal cycler:
   a. Choose the preheat lid option and set to 100°C
   b. Set the reaction volume to 60 µl
   c. 37°C for 15 minutes
   d. 55°C for 15 minutes
   e. Hold at 4°C

   Total program time is ~35 minutes.

Procedure

1. In a 1.7 ml tube, combine exactly the following volumes to prepare Rapid Master Mix. Multiply each volume by the number of samples.
   - CBM (29.7 µl)
   - CEM (2.9 µl)
   - LRA (3.7 µl)
   - EBLTS (11 µl)

   Reagent overage is included in the volumes.
2. Pipette Rapid Master Mix to resuspend.
3. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
4. Add 43 µl Rapid Master Mix to each well.
5. Pipette 10 times to mix.
6. Seal and then centrifuge at 280 × g for 10 seconds.
7. Place on the preprogrammed thermal cycler and run the RLP program.
8. Centrifuge at 280 × g for 10 seconds, and then unseal.
9. Add 10 µl ST2 to each well.
10. Seal and shake at 2200 rpm for 1 minute.
11. Incubate at room temperature for 5 minutes.
12. Centrifuge at 280 × g for 10 seconds, and then unseal.
13. Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
14. Remove and discard all supernatant.
15. Wash beads as follows.
    a. Remove from the magnetic stand.
    b. Add 100 µl TWB2 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.

16. Repeat steps a–e to wash beads a **second** time. Retain the supernatant.
TWB2 remains in the wells to prevent overdrying.
17. Keep on the magnetic stand and proceed immediately to *Amplify and Add Indexes* on page 15.

Amplify and Add Indexes

This step uses PCR to selectively amplify the tagmented cDNA fragments and add indexes and primer sequences for cluster generation.

Consumables

- EPM (Enhanced PCR Mix)
- Index Adapters (UDP0XXX)
- 1.7 ml microcentrifuge tube, RNase-free
- 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.

Preparation

1. Prepare the following consumables:
   - EPM—Invert to mix, and then centrifuge briefly.
   - UDP0XXX—Vortex to mix, and then centrifuge at 1000 × g for 1 minute.
2. Save the following RLP_PCR program on the thermal cycler:
   a. Choose the preheat lid option and set to 100°C
   b. Set the reaction volume to 50 µl
   c. 72°C for 3 minutes
   d. 98°C for 30 seconds
   e. X cycles of:
      - 98°C for 10 seconds

- 60°C for 30 seconds
- 72°C for 30 seconds

f. 72°C for 3 minutes

g. Hold at 4°C for ≤ 16 hours

Total program time is ~30–45 minutes.

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

1. Combine the following volumes to prepare PCR Master Mix. Multiply each volume by the number of samples.
   - EPM (23 µl)
   - Nuclease-free ultrapure water (23 µl)

   Reagent overage is included in the volumes.
2. Vortex PCR Master Mix to mix.
3. Keep the plate on the magnetic stand. Remove and discard all TWB2 supernatant from each well.
4. Use a 20 µl pipette to remove all residual supernatant.
   Foam that remains on the well walls is normal and does not affect the library.
5. Remove the plate 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. Transfer 10 µl UDP0XXX from each well of the index adapter plate to each well of 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 RLP_PCR 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

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 12 Room Temperature Storage

Reagent	Box Name	Instructions
IPB	Illumina DNA/RNA Prep Tag Buffers 96 v2	Use at room temperature.

Table 13 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 library.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)
- 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:
   - IPB—Vortex and invert to mix.
   - RSB—Vortex and invert to mix.
2. 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. Vortex IPB to resuspend.
5. Add 81 µl IPB to each well containing a sample.
6. Mix using one of the following methods:
   - Seal and shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
   - Slowly pipette to mix until beads are resuspended.
7. Incubate at room temperate for 5 minutes.
8. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
9. Remove and discard all supernatant.
10. Wash beads as follows.
    a. Keep on the magnetic stand and add 175 µl fresh 80% EtOH to each well.
    b. Wait 30 seconds.
    c. Remove and discard all supernatant from each well.
11. Wash beads a **second** time.
12. Using a 20 µl pipette, remove all residual EtOH.
13. Air-dry on the magnetic stand for 2 minutes.

    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 17 µl RSB to each well.
16. Mix using one of the following methods:
    - Seal the plate and shake at 2200 rpm for 1 minute.
    - Slowly pipette until the beads are resuspended, and then seal.
17. If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
18. Incubate at room temperature for 2 minutes.
19. Centrifuge at 280 × g for 10 seconds.
20. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
21. Transfer 15 µl supernatant from each well to the corresponding well of 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 14 2°C to 8°C Storage

Reagent	Box Name	Instructions
EHB2	Illumina DNA/RNA Fast Hyb Enrich Beads 96	Bring to room temperature.
ET2	Illumina DNA/RNA Fast Hyb Enrich Beads 96	Bring to room temperature.
SMB4	Illumina DNA/RNA Fast Hyb Enrich Beads 96	Let stand for 30 minutes to bring to room temperature.

Table 15 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EE1	Illumina RNA Single Hyb Enrich PCR 96	Thaw at room temperature.
EEW	Illumina RNA Single Hyb Enrich PCR 96	Thaw at room temperature.
HP3	Illumina RNA Single Hyb Enrich PCR 96	Thaw at room temperature.
NHB2	Illumina RNA Single Hyb Enrich PCR 96	Thaw at room temperature.
Illumina Rapid Viral Surveillance Panel v2	Illumina Rapid Viral Surveillance Panel v2	Thaw on ice.

Pool Library

This step quantifies and normalizes libraries, then combines them into one pool for three-plex enrichment.

Consumables

- 96-well PCR plate, semiskirted

- Qubit dsDNA BR Assay Kit
- **[Optional]** D1000 ScreenTape

Preparation

- Illumina Rapid Viral Surveillance Panel v2—Vortex and invert to mix.

Procedure

1. Analyze 1 µl library with the Qubit dsDNA BR Assay Kit.
   For best results, each library should be > 80 ng/µl.
2. **[Optional]** Analyze 1 µl library with the Agilent TapeStation System and D1000 ScreenTape.
3. In one well of a PCR plate, combine 2.5 ul (x 3) of library for a 3 plex enrichment plexity.

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)
- Illumina Rapid Viral Surveillance Panel v2
- 96-well PCR plate, semiskirted
- Microseal 'B' adhesive film

About Reagents

- NHB2—Precipitates and separates during storage.

Preparation

1. Preheat the microheating system to 50°C.
2. Prepare the following consumables:
   - EHB2:
     a. Vortex to mix.
     b. If crystals and cloudiness are observed, repeat vortex, or pipette up and down to mix well until the solution is clear.
   - NHB2:

   a. Vortex at maximum speed 3 times for 10 seconds each to resuspend.
   b. When at room temperature, preheat to 50°C on a microheating system for 5 minutes.
   c. Centrifuge briefly.
   d. Pipette up and down from the bottom of the tube. If crystals and cloudiness are observed, repeat vortex, or pipette up and down to mix well until the solution is clear.
   e. Keep warm until use to prevent the reformation of precipitates.
- Illumina Rapid Viral Surveillance Panel v2—Vortex to mix.
- 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. Save the following HYB program on the thermal cycler:
   a. Choose the preheat lid option and set to 100°C
   b. Set the reaction volume to 25 µl
   c. 95°C for 5 minutes
   d. 18 cycles of 1 minute each:
      - 94°C for the first cycle
      - Decrease 2°C per subsequent cycle
   e. 58°C for 90 minutes
   f. Hold at 58°C for up to 24 hours

   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 the PCR plate from Pool and Normalize Library *in the order listed*.
   a. RVSP v2 volume (2.5 ul)
   b. NHB2 (12.5 µl)
   c. 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.
6. Proceed immediately to the next procedure before the HYB program hold temperature time ends.

Do not allow the hybridization reactions to cool. Precipitation occurs if the temperature of the hybridization reaction falls below room temperature.

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
     a. Bring to room temperature before use.
     b. 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 16 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EPM	Illumina RNA Single Hyb Enrich PCR 96	Thaw on ice.
PPC	Illumina RNA Single Hyb Enrich PCR 96	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.
   a. Choose the preheat lid option and set to 100°C
   b. Set the reaction volume to 50 µl
   c. 98°C for 30 seconds
   d. 14 cycles of:
      - 98°C for 10 seconds
      - 60°C for 30 seconds
      - 72°C for 30 seconds
   e. 72°C for 5 minutes
   f. 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 17 Room Temperature Storage

Reagent	Box Name	Instructions
IPB	Illumina DNA/RNA Prep Tag Buffers 96 v2	Use at room temperature.

Table 18 2°C to 8°C Storage

Reagent	Box Name	Instructions
RSB	Illumina DNA/RNA Fast Hyb Enrich Beads 96	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)
- Freshly prepared 80% ethanol (EtOH)
- 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:
   - IPB—Vortex and invert to mix.
   - RSB—Vortex and invert to mix.
2. Prepare 80% EtOH from absolute EtOH.

Procedure

1. Centrifuge the sealed plate at 280 × g for 10 seconds.
2. Respend 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 40 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, 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

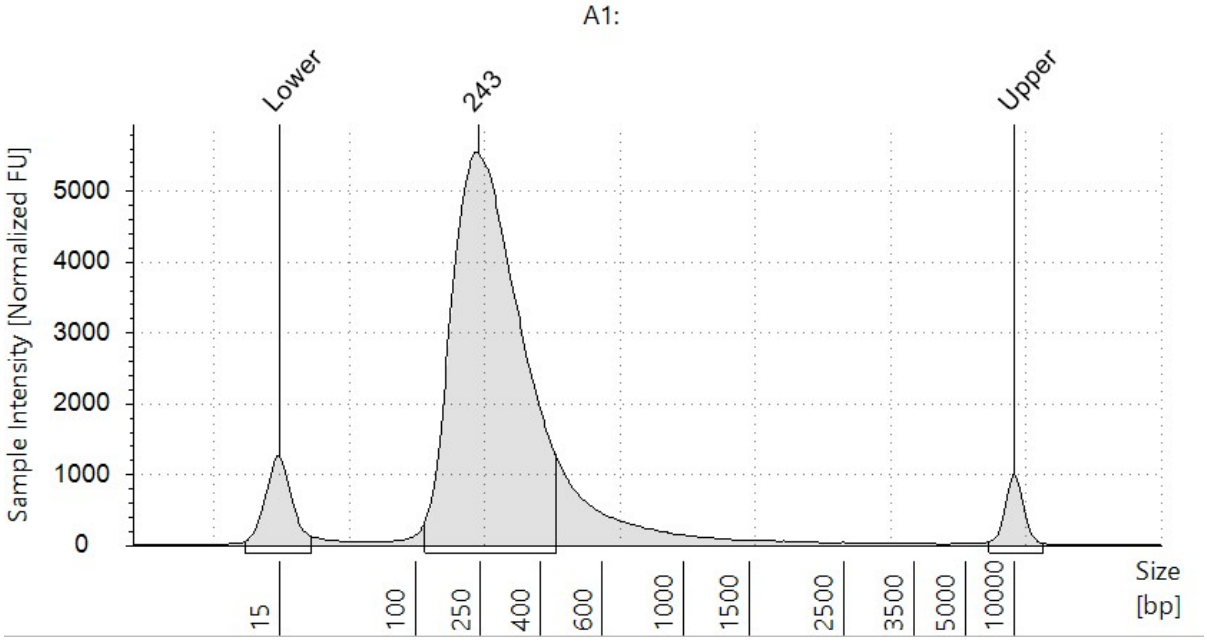
Check the enriched library using both of the following methods:

- 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 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 TapeStation Trace



Dilute Library to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system and is the first step in a serial dilution. 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.

Samples with varying viral titer can generate a wide range in number of on-target reads. Loading concentrations must be optimized based on the sample type.

1. Obtain the molarity value of the library or pooled libraries using the applicable method:
   - For libraries quantified with a Qubit, use the following formula to calculate molarity value. Use the average library size and the concentration from the Qubit.
   - For libraries quantified with a KAPA library quantification qPCR method, use 300 bp as the average library insert size.

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

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
MiSeq i100*	0.8	80
NextSeq 550 and NextSeq 500	20	0.8
NextSeq 1000 and NextSeq 2000	2	750
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 Rapid Viral Surveillance Panel v2 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
200076914 v00	June 2026	Initial release.

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