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Illumina Rapid mRNA Prep

Product Documentation

ILLUMINA PROPRIETARY
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Table of Contents

Overview

The Illumina Rapid mRNA Prep kit converts the messenger (mRNA) in total RNA into up to 384 dual-indexed libraries.

Oligo(dT) magnetic beads purify and capture the mRNA molecules containing polyA tails. The purified mRNA is fragmented and copied into first strand complimentary DNA (cDNA) using reverse transcriptase and random primers. The resulting products are purified and selectively amplified for sequencing on an Illumina system.

The kit offers the following features:

- Capture of both coding RNA and multiple forms of noncoding RNA that are polyadenlyated
- A polyA capture to selectively sequence mRNA
- cDNA synthesis and tagmentation in a single step
- Unique dual (UD) indexing with Illumina DNA/RNA UD Indexes

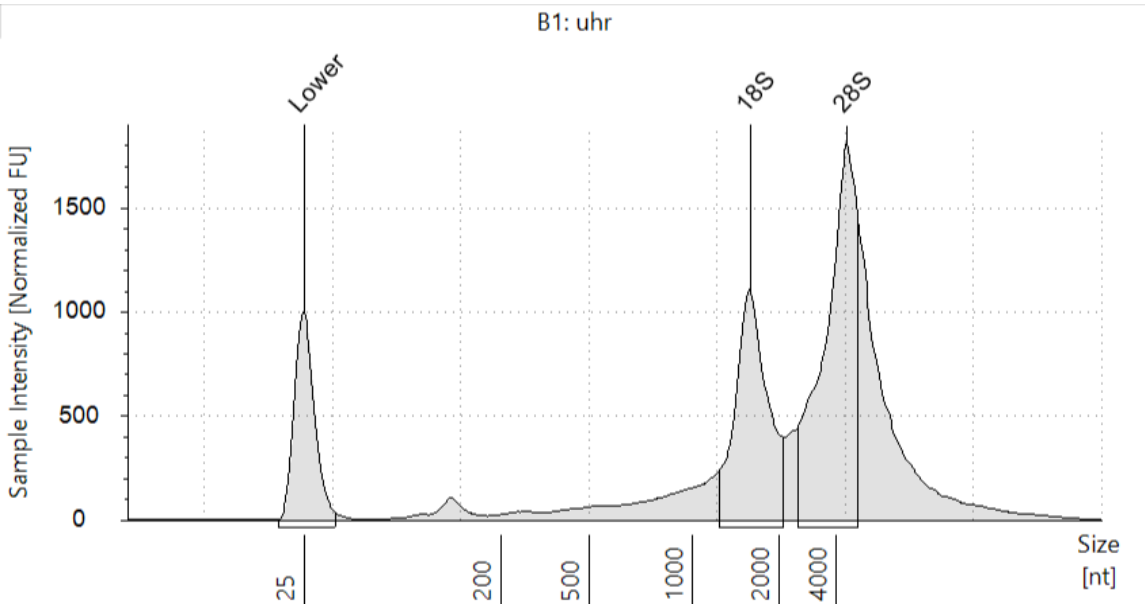
Input Recommendations

The protocol is optimized for 1–1000 ng of high-quality total eukaryotic RNA. Lower input amounts and lesser quality can reduce library yield. Determining the input amount for other species and quality levels requires further optimization.

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.

The following figure provides an example trace for Universal Human Reference (UHR) total RNA.

Figure 1 Example Trace of UHR Input



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 mRNA Prep protocol requires the Illumina mRNA Capture Kit, the Illumina Rapid RNA Prep (S) Tagmentation kit, 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, and library prep in a 16-sample workflow or 96-sample workflow.
- The index sets provide premixed Index 1 (i7) and Index 2 (i5) adapters.

Library Prep Reagents

Catalog Item	Catalog #	Box Name	Box #
Illumina mRNA Capture Kit (16 samples)	20158829	Illumina Poly(A) Capture 16	20040893
Illumina mRNA Capture Kit (96 samples)	20158830	Illumina Poly(A) Capture 96	20040894
Illumina Rapid RNA Prep (S) Tagmentation (16 samples)	20158823	Illumina Rapid RNA Prep	20158860
		Illumina DNA/RNA Prep IPB Tag Buffers v2	20158861
		Illumina DNA/RNA Prep – Tagmentation (S) Beads	20026303
Illumina Rapid RNA Prep (S) Tagmentation (96 samples)	20158824	Illumina Rapid RNA Prep	20115932
		Illumina DNA/RNA Prep IPB Tag Buffers v2	20150313
		Illumina DNA/RNA Prep – Tagmentation (S) Beads	20026214

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

Illumina Rapid mRNA Prep Kit Contents

Illumina Poly(A) Capture Components, Store at 2°C to 8°C

Tube Quantity		Reagent	Description
16 Samples	96 Samples		
1	1	BBB	Bead Binding Buffer
1	1	BWB	Bead Washing Buffer
1	3	ELB	Enrichment Elution Buffer 1
1	4	RPBX	RNA Purification Beads

Illumina Rapid RNA Prep (S) Tagmentation Kit Contents

Illumina Rapid RNA Prep, 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	2	CBM	cDNA Buffer Mix
1	1	CEM	cDNA Enzyme Mix
1	1	LRA	Library Reducing Reagent
1	4	EPM	Enhanced PCR Mix

Illumina DNA/RNA Prep IPB Tag Buffers v2, Store at Room Temperature*

Tube Quantity		Reagent	Description
16 Samples	96 Samples		
1	4	ST2	Stop Tagment Buffer 2
1	1	TWB2	Tagmentation Wash Buffer 2
1	2	IPB	Illumina Purification Beads

*Shipped at 2°C to 8°C.

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

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

Index Adapter Kit Contents

Each kit contains one plate of indexes.

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
96-well twin.tec 250 µl PCR plates, semiskirted	One of the following suppliers: • Thermo Fisher Scientific, catalog # E951020303 • VWR, catalog # 47744-106
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, 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

Consumable	Supplier
Ultrapure water, nuclease-free	General lab 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
[Shaking workflow] BioShake iQ High-Speed Thermal Mixer	Q Instruments, catalog # 1808–0506
[Shaking workflow] BioShake PCR Plate Adapter	Q Instruments, catalog # 1808–1041
Vortexer	General lab supplier
[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 mRNA Prep protocol and provides instructions for preparing 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 3.

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 Illumina Rapid mRNA support pages or the support pages for your system.

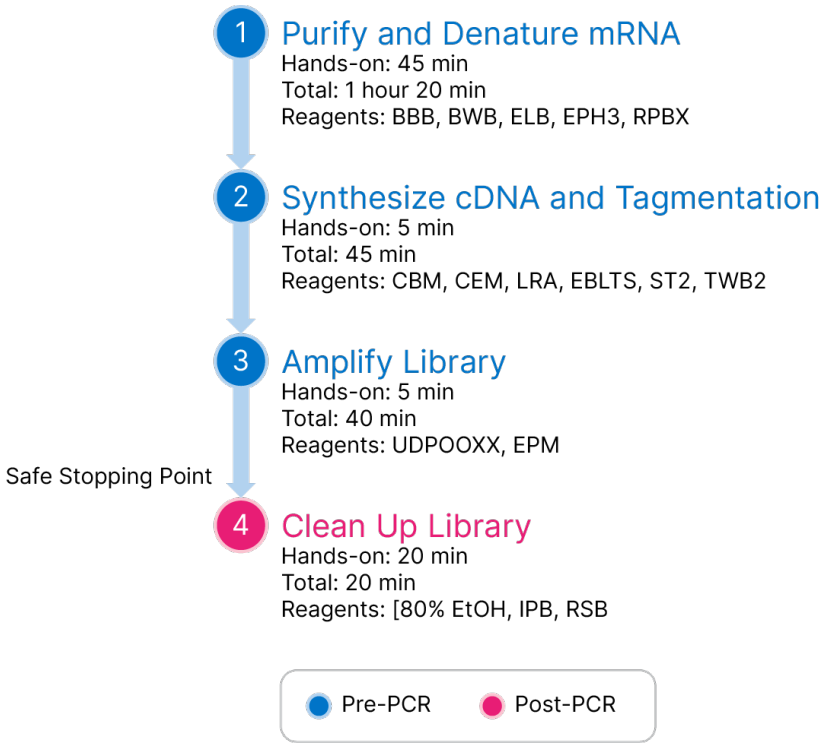
The protocol uses Illumina DNA/RNA UD Indexes to index libraries. These indexes add distinct Index 1 (i7) and Index 2 (i5) sequences to each end of a fragment. Each index sequence is 10 bp long.

- 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 Rapid mRNA Prep Workflow

The following diagram provides an overview of the Illumina Rapid mRNA Prep protocol using a single sample. The safe stopping point is marked between steps.

Figure 2 Rapid mRNA Workflow



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 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 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 1 Room Temperature Storage

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

Table 2 2°C to 8°C Storage

Reagent	Box Name	Instructions
BBB	Illumina Poly(A) Capture	Bring to room temperature.
BWB	Illumina Poly(A) Capture	Let stand for 30 minutes to bring to room temperature.
EBLTS	Illumina DNA/RNA Prep – Tagmentation (S) Beads	Bring to room temperature.

Reagent	Box Name	Instructions
ELB	Illumina Poly(A) Capture	Thaw at room temperature.
RPBX	Illumina Poly(A) Capture	Let stand for 30 minutes to bring to room temperature.

Table 3 -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.
EPM	Illumina Rapid RNA Prep	Thaw at room temperature.
Index Adapter Plate	Illumina DNA/RNA UD Indexes	Thaw at room temperature.
CBM	Illumina Rapid RNA Prep	Thaw at room temperature.
CEM	Illumina Rapid RNA Prep	Thaw at room temperature.

Purify and Denature mRNA

This step uses oligo(dT) magnetic beads to capture messenger RNAs (mRNAs) with polyA tails. The RNA is then denatured and primed for complementary DNA (cDNA) synthesis.

Consumables

- BBB (Bead Binding Buffer)
- BWB (Bead Washing Buffer)
- ELB (Enrichment Elution Buffer 1)
- EPH3 (Elute, Prime, Fragment High Concentration Mix)
- RPBX (RNA Purification Beads)
- Nuclease-free ultrapure water
- 1.7 ml microcentrifuge tube, RNase-free
- 96-well PCR plates, semiskirted (2)
- Microseal 'B' adhesive film

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:
   - BBB—Vortex and invert to mix.
   - BWB—Vortex and invert to mix.
   - ELB—Vortex and invert to mix.
   - EPH3—Vortex to mix, and then centrifuge briefly.
   - RPBX—Vortex to mix.
2. Save the following mRNA_CAP program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Reaction volume is 50 µl
   - 65°C for 5 minutes
   - 4°C for 30 seconds
   - 23° for 5 minutes
   - Hold at 23°C
3. Save the following mRNA_ELT program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Reaction volume is 25 µl
   - 80°C for 2 minutes
   - Hold at 25°C
4. Save the following DEN94_2 program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Reaction volume is 19 µl
   - 94°C for 2 minutes
   - Hold at 4°C

Procedure

Capture mRNA

1. In each well of a new PCR plate, dilute 1–1000 ng total RNA in nuclease-free ultrapure water to a volume of 25 µl.
2. Vortex RPBX to resuspend.
3. Add 25 µl RPBX to each well.
4. 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.
   - Pipette 10 times to mix, and then seal.
5. If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
6. Place on the preprogrammed thermal cycler and run the mRNA_CAP program.
   Total program time is ~15 minutes and each well contains a volume of 50 µl.

Elute mRNA

1. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
2. Place on the magnetic stand and wait 2 minutes.
3. Remove and discard all supernatant.
4. Remove from the magnetic stand.
5. Add 100 µl BWB to each well.
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.
   - Pipette 10 times.
7. Place on the magnetic stand and wait 2 minutes.
8. Remove and discard all supernatant.
9. With a 20 µl pipette, remove all residual BWB.
10. Remove from the magnetic stand.
11. Add 25 µl ELB to each well.
12. Mix using one of the following methods:
    - Seal and shake at 2000 rpm for 1 minute.
    - Pipette to mix until beads are resuspended, and then seal.
13. If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
14. Centrifuge at 280 × g for 10 seconds.
15. Place on the preprogrammed thermal cycler and run the mRNA_ELT program.

Total program time is ~6 minutes and each well contains a volume of 25 µl.

Clean Up mRNA

1. In a 1.7 ml tube on ice, combine exactly the following volumes to prepare Fragmentation Master Mix. Multiply each volume by the number of samples.
   - Nuclease-free ultrapure water (10.5 µl)
   - EPH3 (10.5 µl)

   Volumes include reagent overage for accurate pipetting.
2. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
3. Add 25 µl BBB to each well.
4. Mix using one of the following methods:
   - Seal and shake at 2000 rpm for 1 minute.
   - Pipette to mix until beads are resuspended, and then seal.
5. If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
6. Incubate at room temperature for 5 minutes.
7. Place on the magnetic stand and wait 2 minutes.
8. Remove and discard 50 µl supernatant.
9. Remove from the magnetic stand.
10. Add 100 µl BWB to each well.
11. 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.
    - Pipette 10 times.
12. Place on the magnetic stand and wait 2 minutes.
13. Remove and discard all supernatant.
14. With a 20 µl pipette, remove all residual BWB.
15. Remove from the magnetic stand.
16. Thoroughly pipette Fragmentation Master Mix to mix.
17. Add 19 µl Fragmentation Master Mix to each well.
18. Mix using one of the following methods:
    - Seal and shake at 2000 rpm for 1 minute.
    - Pipette to mix until beads are resuspended, and then seal.
19. If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
20. Incubate at room temperature for 2 minutes.

21. Centrifuge at 280 × g for 10 seconds.

Denature mRNA

1. Place on the preprogrammed thermal cycler and run the DEN94_2 program.
   Total program time is ~5 minutes and each well contains a volume of 19 µl.
2. Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
3. Place on the magnetic stand and wait 2 minutes.
4. Transfer 17 µl supernatant from each well to a new PCR plate.
5. Set aside the new PCR plate on ice.

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 Reagent)
- ST2 (Stop Tagment Buffer 2)
- TWB2 (Tagmentation Wash Buffer 2)
- 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 19.

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 Adapter Plate (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 Amount (ng)	Number of Cycles (X)
1	18
10	15
25	15
100	12
1000	10

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 4 Room Temperature Storage

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

Table 5 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 45 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 30 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.

Check Libraries

Perform the following procedures to check the concentration and quality of the libraries. For intact RNA samples, the average fragment length is ~300-400 bp. The expected insert size is ~140 bp.

1. Analyze 2 µl library using the Qubit dsDNA BR or HS Assay Kit.
   Use the HS kit for low input samples. The HS kit may require a 1:5–1:10 dilution based on expected yield.
2. **[Optional]** Analyze 4 µl 1:10,000 diluted library using the KAPA qPCR Library Quantification Kit for further quantification.

The following figures provide example trace outputs for a final library generated from 10 ng and 25 ng Universal Human Reference (UHR) RNA input.

Figure 3 TapeStation Trace (10 ng of Total RNA)

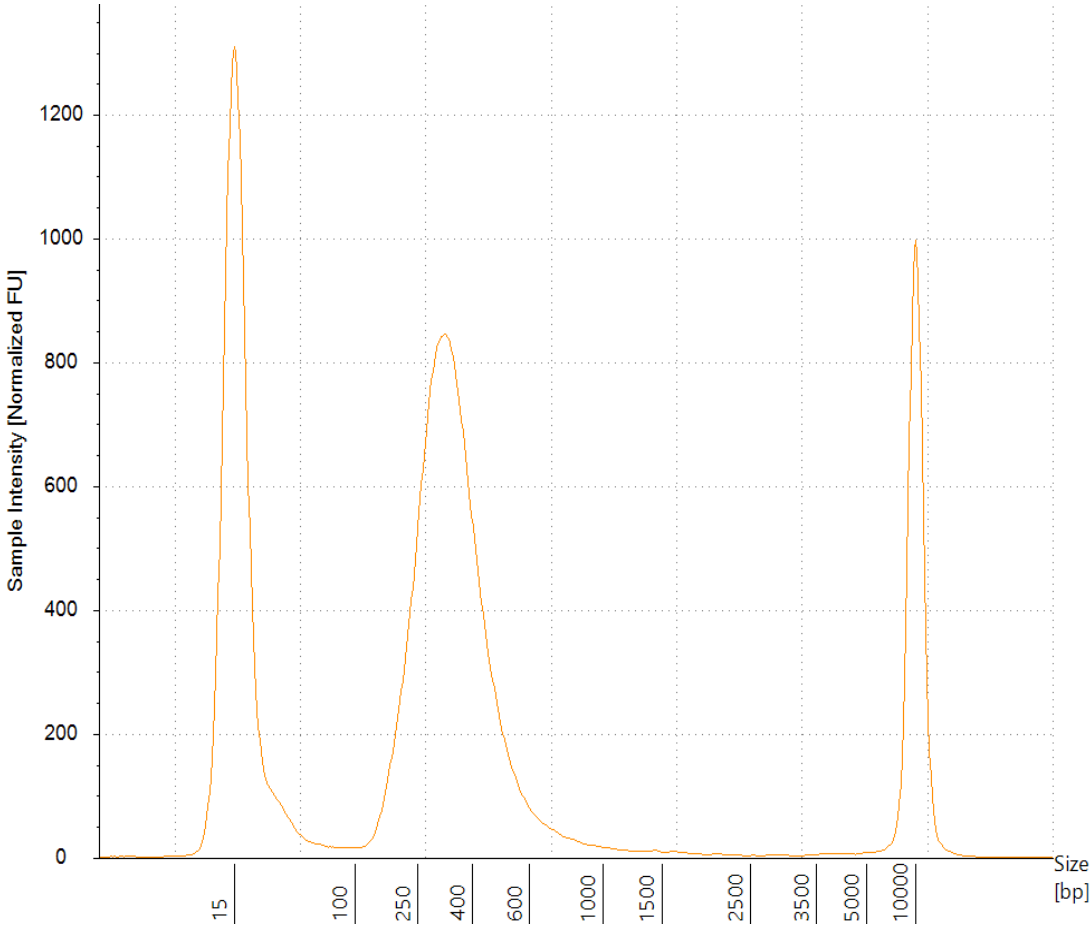
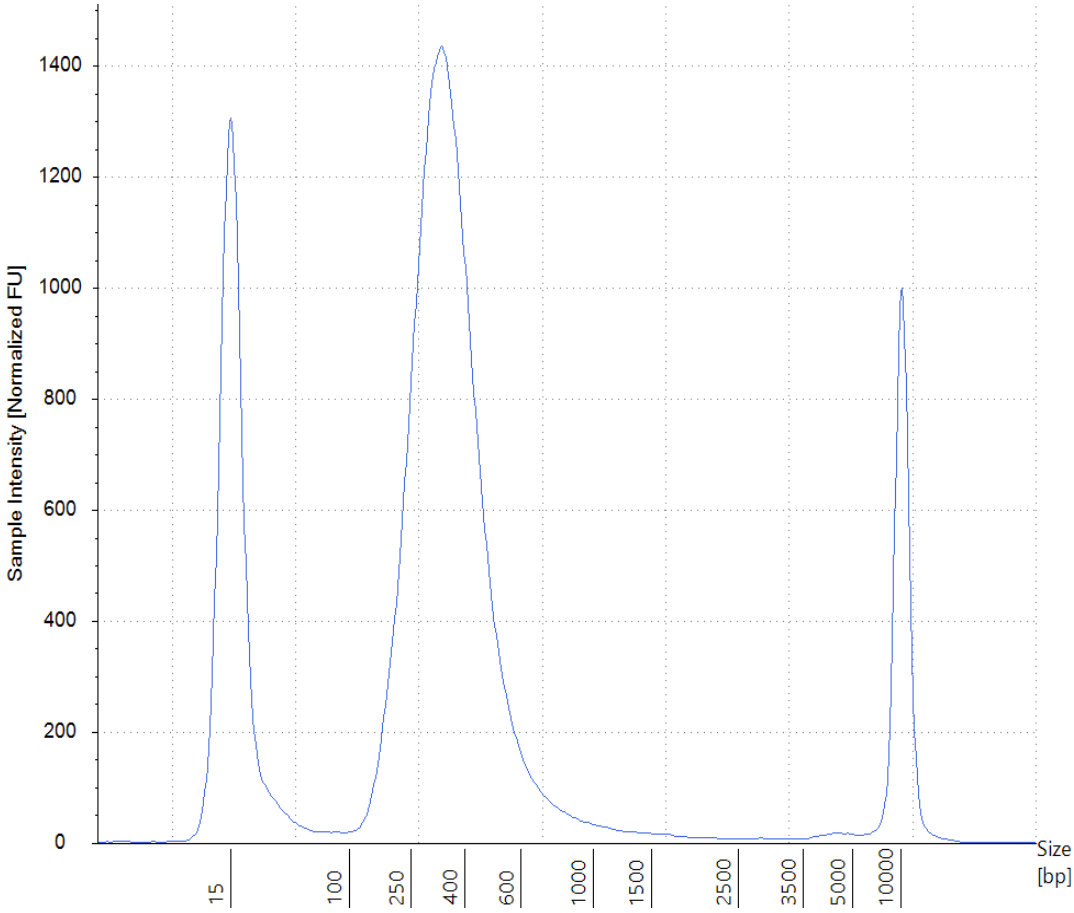


Figure 4 TapeStation Trace (25 ng of Total RNA)



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.

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)
NextSeq 550 and NextSeq 500	1	1.1–1.4
NextSeq 1000 and NextSeq 2000*	2	750
NovaSeq 6000	0.5	100
NovaSeq X	4	150

* For the NextSeq 1000 and NextSeq 2000 systems, use the RSB with Tween 20 supplied with the system to dilute below 10 nM.

3. Dilute each library to the starting concentration for your system using RSB. Add 10 µl each diluted library to 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 mRNA Prep 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 # 200077001 v00	June 2026	Initial release.

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