

Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus

Reference Guide



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Revision History

Document	Date	Description of Change
Document # 1000000124514 v01	August 2020	Removed 1-10 ng input from PCR amplification table and replaced with individual rows for 1 ng and 10 ng inputs. Updated workflow diagram description to include the number of samples used to calculate processing times. Corrected formatting of index kit names.
Document # 1000000124514 v00	June 2020	Initial release.

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Chapter 1 Overview

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Introduction

The Illumina® Stranded Total RNA Prep, Ligation with Ribo-Zero Plus kit converts total RNA into up to 384 dual-indexed libraries. Depletion reagents bind and deplete abundant transcripts from total RNA. Reverse transcription then converts the remaining RNA into cDNA, while subsequent ligation and amplification steps add adapters for clustering and sequencing on an Illumina system.

The kit offers the following features:

- ▶ An enzymatic depletion method that removes abundant transcripts from total RNA
- ▶ Library prep for analysis of coding and multiple forms of noncoding RNA
- ▶ Strand information on RNA transcripts
- ▶ Unique dual (UD) indexing with the IDT for Illumina RNA UD Indexes

RNA Input Recommendations

The protocol is optimized for 10–100 ng RNA input. It accepts 1–1000 ng purified total RNA input from high-quality RNA samples (RIN \geq 9) and accepts 10–100 ng RNA input from low-quality RNA (RIN \geq 2) samples or FFPE (DV200 > 55%) samples. Library performance can vary with lower input amounts and lesser quality RNA.

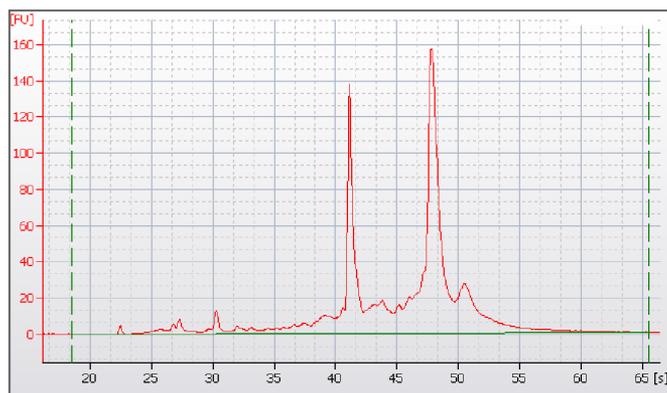
The protocol can deplete rRNA from the following species.

Species	RNA Type
Bacteria	<i>Escherichia coli</i> (gram-negative) or <i>Bacillus subtilis</i> (gram-positive) total RNA*
Globin	Total RNA isolated from human blood
Human	Universal Human Reference (UHR) RNA
Mouse	Universal Mouse Reference RNA
Rat	Universal Rat Reference RNA

*Performance can vary when using other bacterial species.

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 input. The trace is from a 2100 Bioanalyzer System using an RNA 6000 Pico Kit.

Figure 1 Example Trace of UHR Input

Additional Resources

The following resources provide instructions and guidelines for using the Illumina Stranded Total RNA kit to prepare libraries. Visit the Illumina Stranded Total RNA support pages for additional information:

- ▶ [Compatible products and requirements for recording sample information, sequencing libraries, and analyzing data](#)
- ▶ [Questions and answers about using the kit](#)
- ▶ [Courses for related products and subjects](#)
- ▶ [The latest versions of the kit documentation](#)

Resource	Description
Custom Protocol Selector	A tool for generating end-to-end instructions tailored to your library prep method, run parameters, and analysis method, with options to refine the level of detail.
Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus Checklist (document # 1000000124515)	Provides a checklist of steps for the experienced user.
Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus Consumables & Equipment (document # 1000000124517)	Provides an interactive checklist of user-provided consumables and equipment.
Index Adapters Pooling Guide (document # 1000000041074)	Provides guidelines for preparing dual-indexed libraries with balanced index combinations for sequencing on Illumina systems.
Illumina Adapter Sequences (document # 1000000002694)	Provides the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.

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Introduction

This section describes the Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus protocol with step-by-step instructions.

- ▶ 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, including library prep reagents and index anchors and adapters. For a complete list, see *Supporting Information on page 21*.

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, see the Illumina Stranded Total RNA support pages or the support pages for your system.

The protocol uses IDT for Illumina RNA UD Indexes to index libraries in two separate steps, first adding anchors and then the UD indexes. The UD index primers 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, see the *Index Adapters Pooling Guide (document # 1000000041074)*.
- ▶ For index adapter sequences and how to record them, see *Illumina Adapter Sequences (document # 11000000002694)*.

Handling Beads

The protocol uses two types of beads: Agencourt RNAClean XP and Agencourt AMPure XP. Each bead type has a specific technical application and cannot be substituted.

Apply the following techniques when handling beads:

- ▶ Use beads at room temperature.
- ▶ **Do not** use beads that have been stored < 2°C.

- ▶ Vortex beads before each use and frequently throughout the protocol to resuspend. Resuspended beads are evenly distributed and homogenous in color.
- ▶ Dispense liquid directly onto bead pellets so that beads on the side of the wells are wetted.
- ▶ 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).
- ▶ If beads adhere to well walls, centrifuge at $280 \times g$ for 3 seconds, and then pipette to resuspend.

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 1 year.
 - ▶ If you must reuse the sample, aliquot 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.

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.

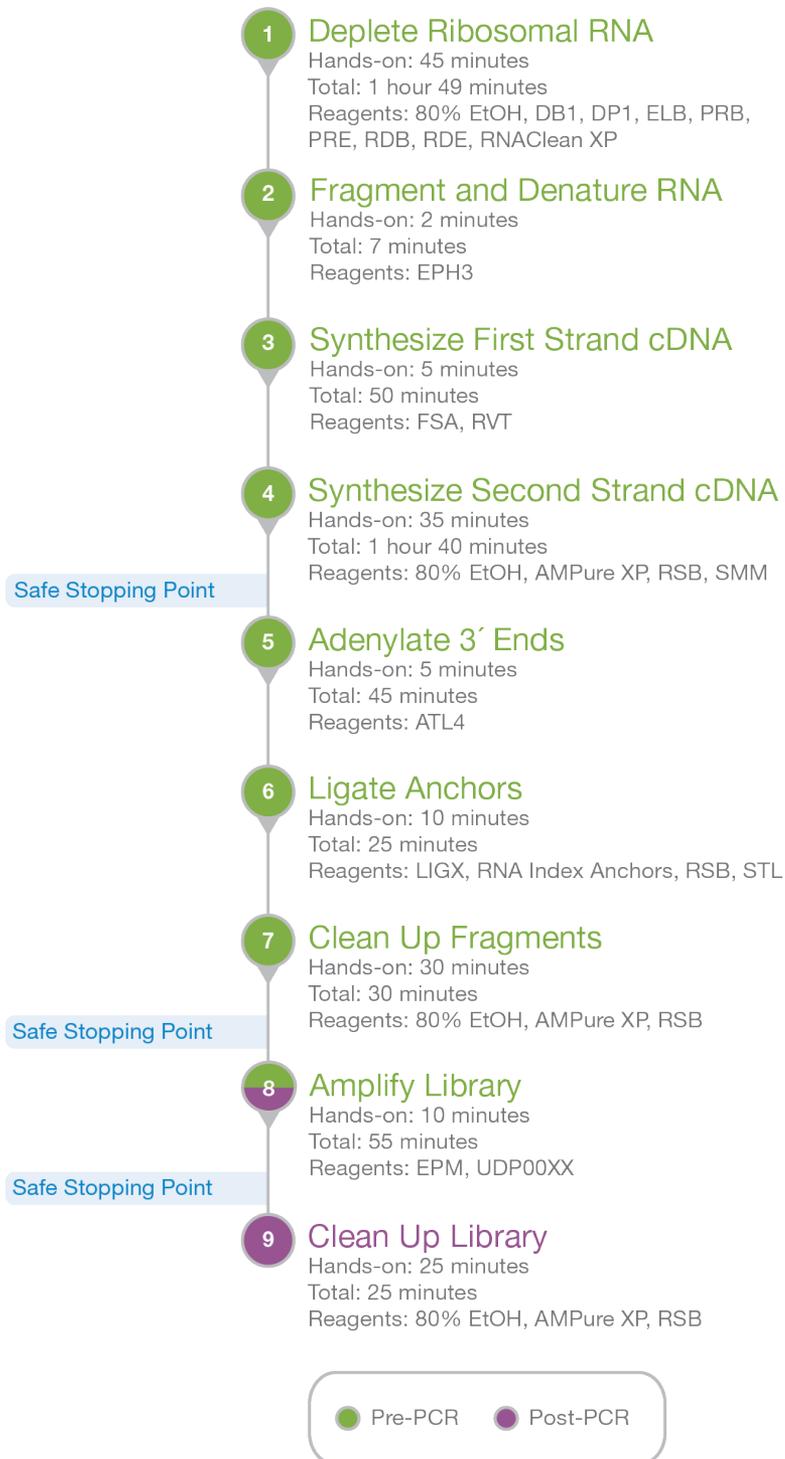
Mixing and Centrifugation

- ▶ The protocol includes the choice to mix by shaking or pipetting. Use the applicable magnetic stand:
 - ▶ If you choose to mix by pipette, use a Magnetic Stand-96.
 - ▶ If you choose to mix by shaking, use a DynaMag-96 Side Magnet.

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

Library Prep Diagram

The following diagram provides an overview of the Illumina Stranded Total RNA protocol when using 24 samples. Safe stopping points are marked between steps.



Deplete rRNA

This step depletes abundant rRNA from purified total RNA. DNA probes bind to rRNA targets, which are both enzymatically-digested to produce total RNA free from abundant transcripts.

RNA-specific magnetic beads then recover and purify the rRNA-depleted RNA. The purification removes any remaining salts and buffers.

Consumables

- ▶ DB1 (Depletion Probe Buffer) (blue cap)
- ▶ DP1 (Depletion Probe Pool) (blue cap)
- ▶ ELB (Elution Buffer)
- ▶ PRB (Probe Removal Buffer) (red cap)
- ▶ PRE (Probe Removal Enzyme) (red cap)
- ▶ RDB (RNA Depletion Buffer) (yellow cap)
- ▶ RDE (RNA Depletion Enzyme) (yellow cap)
- ▶ Agencourt RNAClean XP
- ▶ Freshly prepared 80% ethanol (EtOH)
- ▶ Nuclease-free ultrapure water
- ▶ 1.7 ml microcentrifuge tubes, RNase-free (3)
- ▶ 96-well PCR plates, semiskirted (2)
- ▶ Microseal 'B' adhesive film
- ▶ Prepare for a later procedure:
 - ▶ EPH3 (Elute, Prime, Fragment High Mix)
 - ▶ FSA (First Strand Synthesis Act D Mix)

About Reagents

- ▶ Prepare 80% EtOH fresh and discard after one day. The protocol has four cleanup steps that require fresh 80% EtOH.

Preparation

- 1 Prepare the following consumables:

Reagent	Storage	Instructions
DB1	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
DP1	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
ELB	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
EPH3	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
FSA	-25°C to -15°C	Thaw on ice. Vortex to mix, and then centrifuge briefly.
PRB	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
PRE	-25°C to -15°C	Store until needed. Flick to mix, and then centrifuge briefly.
RDB	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
RDE	-25°C to -15°C	Store until needed. Flick to mix, and then centrifuge briefly.

Reagent	Storage	Instructions
RNAClean XP	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.

- 2 Prepare 80% EtOH from absolute EtOH.
- 3 Save the following HYB_DP1 program on the thermal cycler:
 - ▶ Choose the preheat lid option and set to 100°C
 - ▶ Reaction volume is 15 µl
 - ▶ 95° for 2 minutes
 - ▶ Decrease 0.1°C per second until the temperature reaches 37°C
 - ▶ Hold at 37°C
- 4 Save the following RNA_DEP program on the thermal cycler:
 - ▶ Choose the preheat lid option and set to 100°C
 - ▶ Reaction volume is 20 µl
 - ▶ 37°C for 15 minutes
 - ▶ Hold at 4°C
- 5 Save the following PRB_REM program on the thermal cycler:
 - ▶ Choose the preheat lid option and set to 100°C
 - ▶ Reaction volume is 30 µl
 - ▶ 37°C for 15 minutes
 - ▶ 70°C for 15 minutes
 - ▶ Hold at 4°C

Procedure

Hybridize Probes

- 1 Using nuclease-free ultrapure water, dilute 1–1000 ng total RNA to a volume of 11 µl in each well of a new PCR plate.
- 2 In a 1.7 ml tube on ice, combine exactly the following volumes to prepare Hybridize Probe Master Mix. Multiply each volume by the number of samples.
 - ▶ DB1 (3.6 µl)
 - ▶ DP1 (1.2 µl)
 Volumes include reagent overage for accurate pipetting.
- 3 Thoroughly pipette Hybridize Probe Master Mix to mix.
- 4 Add 4 µl Hybridize Probe Master Mix to each well.
- 5 Pipette 10 times, and then seal.
- 6 Place on the preprogrammed thermal cycler and run the HYB_DP1 program. Total program time is ~15 minutes and each well contains a volume of 15 µl.

Deplete rRNA

- 1 In a 1.7 ml tube on ice, combine exactly the following volumes to prepare rRNA Depletion Master Mix. Multiply each volume by the number of samples.
 - ▶ RDB (4.8 µl)
 - ▶ RDE (1.2 µl)
 Volumes include reagent overage for accurate pipetting.

- 2 Thoroughly pipette rRNA Depletion Master Mix to mix.
- 3 Centrifuge the sealed PCR plate at $280 \times g$ for 10 seconds.
- 4 Add $5 \mu\text{l}$ rRNA Depletion Master Mix to each well.
- 5 Pipette 10 times, and then seal.
- 6 Place on the preprogrammed thermal cycler and run the RNA_DEP program.
Total program time is ~17 minutes and each well contains a volume of $20 \mu\text{l}$.

Remove Probes

- 1 In a 1.7 ml tube on ice, combine exactly the following volumes to prepare Probe Removal Master Mix. Multiply each volume by the number of samples.
 - ▶ PRB ($7.7 \mu\text{l}$)
 - ▶ PRE ($3.3 \mu\text{l}$)Volumes include reagent overage for accurate pipetting.
- 2 Thoroughly pipette Probe Removal Master Mix to mix.
- 3 Centrifuge the sealed PCR plate at $280 \times g$ for 10 seconds.
- 4 Add $10 \mu\text{l}$ Probe Removal Master Mix to each well.
- 5 Pipette 10 times, and then seal.
- 6 Place on the preprogrammed thermal cycler and run the PRB_REM program.
Total program time is ~32 minutes and each well contains a volume of $30 \mu\text{l}$.

Clean Up RNA

- 1 Centrifuge the sealed PCR plate at $280 \times g$ for 10 seconds.
- 2 Vortex RNAClean XP to resuspend.
- 3 Add $60 \mu\text{l}$ RNAClean XP to each well.
- 4 Mix using either method:
 - ▶ Seal and shake at 2000 rpm for 1 minute, and then centrifuge at $280 \times g$ for 10 seconds.
 - ▶ Slowly pipette until the beads are resuspended.
- 5 Incubate at room temperature for 5 minutes.
- 6 Place on the magnetic stand and wait 5 minutes.
- 7 Remove and discard $80 \mu\text{l}$ supernatant.
- 8 Wash beads as follows.
 - a Keep on the magnetic stand and add $175 \mu\text{l}$ fresh 80% EtOH to each well.
 - b Wait 30 seconds.
 - c Remove and discard all supernatant from each well.
- 9 Wash beads a **second** time.
- 10 With a $20 \mu\text{l}$ pipette, remove all residual EtOH.
- 11 Air-dry on the magnetic stand for 2 minutes. Do not over-dry the beads.
- 12 Remove from the magnetic stand.

- 13 Add 10.5 μ l ELB to each well.
- 14 Mix using either method:
 - ▶ Seal the plate and centrifuge at $280 \times g$ for 10 seconds, and then shake at 2200 rpm for 1 minute.
 - ▶ Slowly pipette until the beads are resuspended, and then seal.
- 15 If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
- 16 Incubate at room temperature for 2 minutes.
- 17 Centrifuge at $280 \times g$ for 10 seconds.
- 18 Place on the magnetic stand and wait 2 minutes.
- 19 Transfer 8.5 μ l supernatant to the corresponding well of a new PCR plate.
Small amounts of bead carryover do not affect performance.

Fragment and Denature RNA

This step fragments the purified total RNA and anneals random hexamers, which prime the sample for cDNA synthesis.

Consumables

- ▶ EPH3 (Elute, Prime, Fragment High Mix)

Preparation

- 1 Save the following DEN_RNA program on the thermal cycler:
 - ▶ Choose the preheat lid option and set to 100°C
 - ▶ Reaction volume is 17 μ l
 - ▶ 94°C for 2 minutes
 - ▶ Hold at 4°C

Procedure

- 1 Add 8.5 μ l EPH3 to each well.
- 2 Pipette 10 times, and then seal.
- 3 Place on the preprogrammed thermal cycler and run the DEN_RNA program.
Total program time is ~4 minutes and each well contains a volume of 17 μ l.

Synthesize First Strand cDNA

This step reverse transcribes the hexamer-primed RNA fragments to produce first strand complementary DNA (cDNA). The First Strand Synthesis Act D Mix includes Actinomycin D, which allows RNA-dependent synthesis and improves strand specificity while preventing spurious DNA-dependent synthesis.

Consumables

- ▶ FSA (First Strand Synthesis Act D Mix)
- ▶ RVT (Reverse Transcriptase)
- ▶ 1.7 ml microcentrifuge tube, RNase-free

- ▶ Microseal 'B' adhesive film
- ▶ Prepare for a later procedure:
 - ▶ Agencourt AMPure XP
 - ▶ RSB (Resuspension Buffer)
 - ▶ SMM (Second Strand Marking Master Mix)



WARNING

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, see the SDS at support.illumina.com/sds.html.

Preparation

- 1 Prepare the following consumables:

Reagent	Storage	Instructions
AMPure XP	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
RSB	-25°C to -15°C	Thaw at room temperature. Vortex and invert to mix.
RVT	-25°C to -15°C	Store until needed. Flick to mix, and then centrifuge briefly.
SMM	-25°C to -15°C	Thaw on ice. 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
 - ▶ Reaction volume is 25 µl
 - ▶ 25°C for 10 minutes
 - ▶ 42°C for 15 minutes
 - ▶ 70°C for 15 minutes
 - ▶ Hold at 4°C

Procedure

- 1 In a 1.7 ml tube on ice, combine exactly the following volumes to prepare First Strand Synthesis Master Mix. Multiply each volume by the number of samples.
 - ▶ FSA (9 µl)
 - ▶ RVT (1 µl)
 Volumes include reagent overage for accurate pipetting.
- 2 Thoroughly pipette First Strand Synthesis Master Mix to mix.
- 3 Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
- 4 Add 8 µl First Strand Synthesis Master Mix to each well.
- 5 Pipette 10 times, and then seal.
- 6 Place on the preprogrammed thermal cycler and run the FSS program. Total program time is ~43 minutes and 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. In place of deoxythymidine triphosphate (dTTP), deoxyuridine triphosphate (dUTP) is incorporated to quench the second strand during amplification and achieve strand specificity.

Consumables

- ▶ RSB (Resuspension Buffer)
- ▶ SMM (Second Strand Marking Master Mix)
- ▶ Agencourt AMPure XP
- ▶ Freshly prepared 80% ethanol (EtOH)
- ▶ 96-well PCR plate, semiskirted
- ▶ Microseal 'B' adhesive film

Preparation

- 1 Save the following SSS program on the thermal cycler:
 - ▶ Choose the preheat lid option and set to 40°C
 - ▶ Reaction volume is 50 μ l
 - ▶ 16°C for 1 hour
 - ▶ Hold at 4°C for \leq 16 hours

Procedure

Generate cDNA

- 1 Centrifuge the sealed PCR plate at 280 \times g for 10 seconds.
- 2 Add 25 μ l SMM to each well.
- 3 Pipette 10 times, and then seal.
- 4 Place on the preprogrammed thermal cycler and run the SSS program. Total program time is ~1 hour and each well contains a volume of 50 μ l.

Clean Up cDNA

- 1 Centrifuge the sealed PCR plate at 280 \times g for 10 seconds.
- 2 Vortex AMPure XP to resuspend.
- 3 Add 90 μ l AMPure XP to each well.
- 4 Mix using either method:
 - ▶ Seal and shake at 2000 rpm for 1 minute, and then centrifuge at 280 \times g for 10 seconds.
 - ▶ Pipette 10 times, and then seal.
- 5 Incubate at room temperature for 5 minutes.
- 6 Place on the magnetic stand and wait 5 minutes.
- 7 Remove and discard 130 μ l supernatant.

- 8 Wash beads as follows.
 - a Keep on the magnetic stand and add 175 μ l fresh 80% EtOH to each well.
 - b Wait 30 seconds.
 - c Remove and discard all supernatant.
- 9 Wash beads a **second** time.
- 10 With a 20 μ l pipette, remove all residual EtOH.
- 11 Air-dry on the magnetic stand for 2 minutes. Do not over-dry the beads.
- 12 Remove from the magnetic stand.
- 13 Add 19.5 μ l RSB to each well.
- 14 Mix using either method:
 - ▶ Seal and shake at 2200 rpm for 1 minute.
 - ▶ Slowly pipette until the beads are resuspended, and then seal.
- 15 If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
- 16 Incubate at room temperature for 2 minutes.
- 17 Centrifuge at 280 \times g for 10 seconds.
- 18 Place on the magnetic stand and wait 2 minutes.
- 19 Transfer 17.5 μ l supernatant to the corresponding well of 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.

Adenylate 3' Ends

This step adds an adenine (A) nucleotide to the 3' ends of the blunt fragments to prevent them from ligating to each other during adapter ligation. A corresponding thymine (T) nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment.

Consumables

- ▶ ATL4 (A-Tailing Mix)
- ▶ Microseal 'B' adhesive film
- ▶ Prepare for a later procedure:
 - ▶ Anchor plate (RNA Index Anchors)
 - ▶ STL (Stop Ligation Buffer)

Preparation

- 1 Prepare the following consumables:

Reagent	Storage	Instructions
Anchor plate	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
ATL4	-25°C to -15°C	Thaw at room temperature. Flick to mix, and then centrifuge briefly.
STL	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.

- Save the following ATAIL program on the thermal cycler:
 - ▶ Choose the preheat lid option and set to 100°C
 - ▶ Reaction volume is 30 µl
 - ▶ 37°C for 30 minutes
 - ▶ 70°C for 5 minutes
 - ▶ Hold at 4°C

Procedure

- If you are resuming the protocol after a safe stopping point, centrifuge the sealed PCR plate at 280 × g for 10 seconds.
- Add 12.5 µl ATL4 to each well.
- Using a 200 µl pipette, pipette 10 times to mix, and then seal.
- Place on the preprogrammed thermal cycler and run the ATAIL program.
Total program time is ~38 minutes and each well contains a volume of 30 µl.

Ligate Anchors

This step ligates pre-index anchors to the ends of the double-stranded cDNA fragments to prepare them for dual indexing. A subsequent PCR amplification step adds the index adapter sequences.

Consumables

- ▶ Anchor plate (RNA Index Anchors) (green plate)
- ▶ LIGX (Ligation Mix)
- ▶ STL (Stop Ligation Buffer)
- ▶ RSB (Resuspension Buffer)
- ▶ Microseal 'B' adhesive film
- ▶ Prepare for a later procedure:
 - ▶ Agencourt AMPure XP

About Reagents

- ▶ Although stored at -25°C to -15°C, LIGX remains liquid and does not require thawing.
- ▶ Each well of the anchor plate is single-use and contains RNA Index Anchors. Anchor plate wells contain the same content and can be used in any order.
- ▶ The anchor plate is green and is distinct from the index adapter plate, which is colorless and used in a later step. Check the plate labels to make sure you are using the correct plate.

Preparation

- Prepare the following consumables:

Reagent	Storage	Instructions
AMPure XP	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
LIGX	-25°C to -15°C	Store until needed. Flick to mix, and then centrifuge briefly.
RSB	-25°C to -15°C	Thaw at room temperature. Vortex and invert to mix.

- Save the following LIG program on the thermal cycler:
 - ▶ Choose the preheated lid option and set to 100°C
 - ▶ Reaction volume is 38 µl
 - ▶ 30°C for 10 minutes
 - ▶ Hold at 4°C

Procedure

Add Anchors

- Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
- Add the following volumes to each well *in the order listed*. Transfer RNA Index Anchors from the anchor plate to the PCR plate.

Order of Addition	Reagent	Volume for Sample Input ≤ 100 ng (µl)	Volume for Sample Input > 100 ng (µl)
1	RSB	2.5	0
2	RNA Index Anchors	2.5	5
3	LIGX	2.5	2.5

- Using a 200 µl pipette, pipette 10 times to mix, and then seal.
- Place on the preprogrammed thermal cycler and run the LIG program.
Total program time is ~13 minutes and each well contains a volume of 38 µl.

Stop Ligation

- Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
- Add 5 µl STL to each well.
- Pipette 15 times to mix.
Each well contains a volume of 43 µl.

Clean Up Fragments

This step uses magnetic beads to purify the adapter-ligated fragments.

Consumables

- ▶ RSB (Resuspension Buffer)
- ▶ Agencourt AMPure XP
- ▶ Freshly prepared 80% ethanol (EtOH)
- ▶ 96-well PCR plate, semiskirted
- ▶ Microseal 'B' adhesive film

Procedure

- 1 Vortex AMPure XP to resuspend.
- 2 Add 34 μ l AMPure XP to each well.
- 3 Mix using either method:
 - ▶ Seal and shake at 2000 rpm for 1 minute, and then centrifuge at $280 \times g$ for 10 seconds.
 - ▶ Slowly pipette until the beads are resuspended.
- 4 Incubate at room temperature for 5 minutes.
- 5 Place on the magnetic stand and wait 5 minutes.
- 6 Remove and discard 67 μ l supernatant.
- 7 Wash beads as follows.
 - a Keep on the magnetic stand and add 175 μ l fresh 80% EtOH to each well.
 - b Wait 30 seconds.
 - c Remove and discard all supernatant.
- 8 Wash beads a **second** time.
- 9 With a 20 μ l pipette, remove all residual EtOH.
- 10 Air-dry on the magnetic stand for 2 minutes. Do not over-dry the beads.
- 11 Remove from the magnetic stand.
- 12 Add 22 μ l RSB to each well.
- 13 Mix using either method:
 - ▶ Seal and shake at 2200 rpm for 1 minute.
 - ▶ Slowly pipette until the beads are resuspended, and then seal.
- 14 If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
- 15 Incubate at room temperature for 2 minutes.
- 16 Centrifuge at $280 \times g$ for 10 seconds.
- 17 Place on the magnetic stand and wait 2 minutes.
- 18 Transfer 20 μ l supernatant to the corresponding well of a new PCR plate. Amplification PCR cycles vary by input amount and quality. When multiple samples are prepared, transfer to separate plates according to the number of PCR cycles specified in the *Amplify Library* procedure.

SAFE STOPPING POINT

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

Amplify Library

This step uses PCR to selectively amplify the anchor-ligated DNA fragments and add indexes and primer sequences for cluster generation. The resulting product is a dual-indexed library: DNA fragments with adapters at each end.

For help with selecting index adapters, see *Pooling Preparation on page 3*.

Consumables

- ▶ EPM (Enhanced PCR Mix)
- ▶ Index adapter plate (UDP0XXX)

About Reagents

- ▶ Each well of the index adapter plate is single-use and contains > 10 μ l 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:

Reagent	Storage	Instructions
EPM	-25°C to -15°C	Thaw at room temperature. Invert to mix, and then centrifuge briefly.
Index adapter plate	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge at 1000 \times g for 1 minute.

- 2 Save the following PCR program on a thermal cycler using the appropriate number of PCR cycles, which are listed in the following table:

- ▶ Choose the preheat lid option and set to 100°C
- ▶ Reaction volume is 50 μ l
- ▶ 98°C for 30 seconds
- ▶ X cycles of:
 - ▶ 98°C for 10 seconds
 - ▶ 60°C for 30 seconds
 - ▶ 72°C for 30 seconds
- ▶ 72°C for 5 minutes
- ▶ Hold at 4°C for \leq 16 hours

Adjust the program to optimize for different input quality and amount.

When multiple samples are amplified on one plate, make sure that the input for each sample is the same.

Input Amount (ng)	Number of PCR Cycles (X) ¹
1	15
10	15
100	13
1000	10

¹ Add two cycles for FFPE or bacterial species.

Total program time is ~44 minutes for 15 cycles, ~39 minutes for 13 cycles, and ~33 minutes for 10 cycles.

Procedure

- 1 If you are resuming the protocol after a safe stopping point, centrifuge the sealed PCR plate at 280 \times g for 10 seconds.

- 2 Using a new pipette tip for each well, pierce the foil covering the index adapter plate wells that you intend to use.
- 3 Add the following volumes to each well of the PCR plate *in the order listed*. Transfer UDPOXXX from the index adapter plate to the PCR plate.
 - ▶ UDPOXXX (10 μ l)
 - ▶ EPM (20 μ l)
- 4 Pipette 10 times to mix, and then seal.
- 5 Place on the preprogrammed thermal cycler and run the PCR program. Each well contains a volume of 50 μ l.

SAFE STOPPING POINT

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

Clean Up Library

This step uses magnetic beads to purify the dual-indexed libraries.

Consumables

- ▶ RSB (Resuspension Buffer)
- ▶ Agencourt AMPure XP
- ▶ Freshly prepared 80% EtOH
- ▶ 96-well PCR plate, semiskirted
- ▶ Microseal 'B' adhesive film

Preparation

- 1 Prepare the following consumables:

Reagent	Storage	Instructions
AMPure XP	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
RSB	-25°C to -15°C	Thaw at room temperature. Vortex and invert to mix.

Procedure

- 1 Centrifuge the sealed PCR plate at 280 \times g for 10 seconds.
- 2 Vortex AMPure XP to resuspend.
- 3 Add 50 μ l AMPure XP to each well.
- 4 Mix using either method:
 - ▶ Seal and shake at 2000 rpm for 1 minute, and then centrifuge at 280 \times g for 10 seconds.
 - ▶ Slowly pipette until the beads are resuspended.
- 5 Incubate at room temperature for 5 minutes.
- 6 Place on the magnetic stand and wait 5 minutes.
- 7 Remove and discard 90 μ l supernatant.
- 8 Wash beads as follows.

- a Keep on the magnetic stand and add 175 μ l fresh 80% EtOH to each well.
 - b Wait 30 seconds.
 - c Remove and discard all supernatant.
- 9 Wash beads a **second** time.
 - 10 With a 20 μ l pipette, remove all residual EtOH.
 - 11 Air-dry on the magnetic stand for 2 minutes. Do not over-dry the beads.
 - 12 Remove from the magnetic stand.
 - 13 Add 17 μ l RSB to each well.
 - 14 Mix using either method:
 - ▶ Seal and shake at 2200 rpm for 1 minute.
 - ▶ Slowly pipette until the beads are resuspended, and then seal.
 - 15 If shaking did not fully resuspend the beads, slowly pipette until the beads are resuspended, and then seal.
 - 16 Incubate at room temperature for 2 minutes.
 - 17 Centrifuge at $280 \times g$ for 10 seconds.
 - 18 Place on the magnetic stand and wait 2 minutes.
 - 19 Transfer 15 μ l supernatant from each well to the corresponding well of a new PCR plate.

SAFE STOPPING POINT

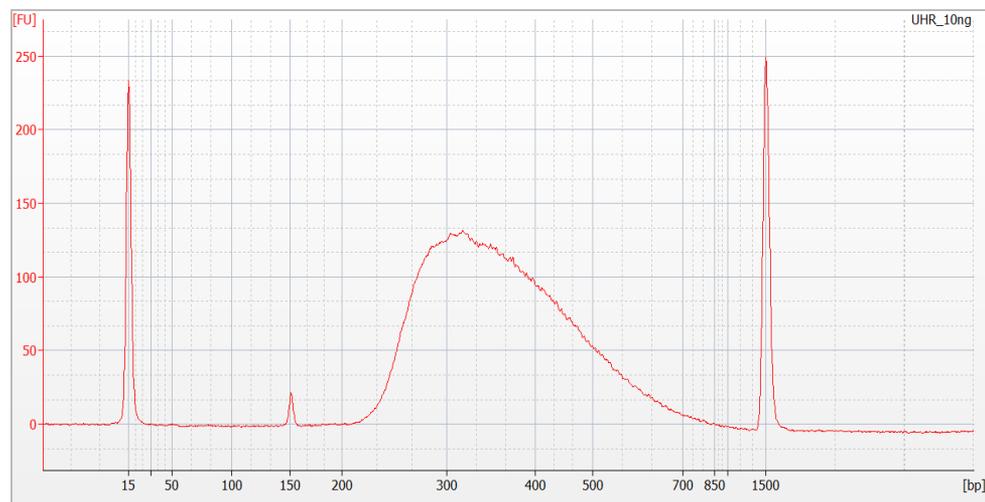
If you are stopping, seal the plate and store at -25°C to -15°C for up to 30 days.

Check Library

This step checks the concentration and quality of the final libraries.

- 1 Analyze 1 μ l library using the Agilent 2100 Bioanalyzer and DNA 1000 Kit.
The following figure provides an example trace for a final library generated from 10 ng Universal Human Reference (UHR) RNA input.

Figure 2 Example Bioanalyzer Trace



- 2 **[Optional]** Analyze 2 µl library using the Qubit dsDNA BR Assay Kit for further quantification. For intact RNA samples, the average fragment length is ~375–475 bp. The expected insert size is ~190 bp.

Dilute Library to the Starting Concentration

This step dilutes libraries to the starting concentration for the NovaSeq 6000, NextSeq 550, or NextSeq 500 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 a Bioanalyzer only, use the molarity value obtained for the library.
 - ▶ For libraries quantified with a Bioanalyzer and Qubit, use the following formula to calculate the molarity value. Apply the average size from the Bioanalyzer and the concentration from the Qubit.

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

- 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
NovaSeq 6000	0.5	100

- 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.

Trim T-Overhang (Optional)

This step trims the first cycle nucleotide at FASTQ generation, removing the T overhang.

Illumina Stranded Total RNA adds a T nucleotide to cDNA inserts, which enables ligation of the insert to the adapter. The addition of the T nucleotide creates low diversity in the first cycle of each insert read, which can result in random base calls at the first sequencing cycle. To improve analysis quality, Illumina recommends trimming the first cycle nucleotide.

- 1 To trim using the FASTQ Toolkit app on BaseSpace Sequence Hub, perform the following actions.
 - a Expand the base trimming settings section.
 - b Enter 1 in the Trim reads at the 5' -end by n positions field.
- 2 To trim using a sample sheet, add the following settings to the sample sheet file.
 - ▶ Read1StartFromCycle,2
 - ▶ Read2StartFromCycle,2



CAUTION

FASTQ generation supports only one setting per run. To ensure correct settings for your libraries, use a separate sample sheet to demultiplex libraries without T-overhang.

Supporting Information

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Introduction

The protocol described in this guide assumes that you have reviewed the contents of this appendix, confirmed kit contents, and obtained all required consumables and equipment.

Acronyms

Acronym	Definition
A	Adenine
ATL4	A-Tailing Mix
cDNA	Complementary DNA
DB1	Depletion Probe Buffer
DP1	Depletion Probe Pool
dsDNA	Double-stranded DNA
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
ELB	Elution Buffer
EPH3	Elute, Prime, Fragment High Concentration Mix
EPM	Enhanced Primer Mix
EtOH	Ethanol
FSA	First Strand Synthesis Act D Mix
LIGX	Ligation Mix
PCR	Polymerase chain reaction
PRB	Probe Removal Buffer
PRE	Probe Removal Enzyme
RDB	RNA Depletion Buffer
RDE	RNA Depletion Enzyme
RIN	RNA Integrity Number
rRNA	Ribosomal RNA
RSB	Resuspension Buffer
RVT	Reverse Transcriptase
SMM	Second Strand Master Mix
STL	Stop Ligation Buffer
T	Thymine
UD	Unique dual
UHR	Universal Human Reference

Kit Contents and Storage

Make sure that you have all reagents identified in this section before starting library prep. The protocol requires one Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus kit and at least one set of IDT for Illumina RNA UD Indexes. Combine all four sets to index up to 384 libraries.

Component	Kit Name	Illumina Catalog #
Library prep	Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus (16 Samples)	20040525
	Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus (96 Samples)	20040529
Indexes	IDT for Illumina RNA UD Indexes Set A, Ligation (96 Indexes, 96 Samples)	20040553
	IDT for Illumina RNA UD Indexes Set B, Ligation (96 Indexes, 96 Samples)	20040554
	IDT for Illumina RNA UD Indexes Set C Ligation (96 Indexes, 96 Samples)	20040555
	IDT for Illumina RNA UD Indexes Set D, Ligation (96 Indexes, 96 Samples)	20040556

The prep kits provides depletion, cDNA synthesis, and library prep reagents in a 16- or 96-sample size. The anchor sets provide premixed Index 1 (i7) and Index 2 (i5) adapters with the adapters necessary for ligation.

The Illumina kits do not include Agencourt RNAClean XP or Agencourt AMPure XP. For suppliers, see [Consumables and Equipment on page 24](#).

Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus (16 Samples) (20040525)

*The Illumina Ribo-Zero Plus rRNA Depletion Kit is available for purchase separately.

Illumina Ribo-Zero Plus rRNA Depletion Kit

Quantity	Reagent	Description	Cap	Shipment	Storage
1	DB1	Depletion Probe Buffer	Blue	-25°C to -15°C	-25°C to -15°C
1	DP1	Depletion Probe Pool	Blue	-25°C to -15°C	-25°C to -15°C
1	ELB	Elution Buffer	Clear	-25°C to -15°C	-25°C to -15°C
1	PRB	Probe Removal Buffer	Red	-25°C to -15°C	-25°C to -15°C
1	PRE	Probe Removal Enzyme	Red	-25°C to -15°C	-25°C to -15°C
1	RDB	RNA Depletion Buffer	Yellow	-25°C to -15°C	-25°C to -15°C
1	RDE	RNA Depletion Enzyme	Yellow	-25°C to -15°C	-25°C to -15°C

Illumina cDNA Synthesis

Quantity	Reagent	Description	Cap	Shipment	Storage
1	EPH3	Elute, Prime, Fragment, High Concentration Mix	Clear	-25°C to -15°C	-25°C to -15°C
1	FSA	First Strand Synthesis Act D Mix	Amber	-25°C to -15°C	-25°C to -15°C
1	RSB	Resuspension Buffer	Clear	-25°C to -15°C	-25°C to -15°C
1	RVT	Reverse Transcriptase	Clear	-25°C to -15°C	-25°C to -15°C
1	SMM	Second Strand Marking Master Mix	Clear	-25°C to -15°C	-25°C to -15°C

Illumina RNA Prep, Ligation

Quantity	Reagent	Description	Cap	Shipment	Storage
1	ATL4	A-Tailing Mix	Clear	-25°C to -15°C	-25°C to -15°C
1	EPM	Enhanced PCR Mix	Clear	-25°C to -15°C	-25°C to -15°C
1	LIGX	Ligation Mix	Clear	-25°C to -15°C	-25°C to -15°C
1	RSB	Resuspension Buffer	Clear	-25°C to -15°C	-25°C to -15°C
1	STL	Stop Ligation Buffer	Red	-25°C to -15°C	-25°C to -15°C

Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus (96 Samples) (20040529)

*The Illumina Ribo-Zero Plus rRNA Depletion Kit is available for purchase separately.

Illumina Ribo-Zero Plus rRNA Depletion Kit

Quantity	Reagent	Description	Cap	Shipment	Storage
1	DB1	Depletion Probe Buffer	Blue	-25°C to -15°C	-25°C to -15°C
1	DP1	Depletion Probe Pool	Blue	-25°C to -15°C	-25°C to -15°C
1	ELB	Elution Buffer	Clear	-25°C to -15°C	-25°C to -15°C
1	PRB	Probe Removal Buffer	Red	-25°C to -15°C	-25°C to -15°C
1	PRE	Probe Removal Enzyme	Red	-25°C to -15°C	-25°C to -15°C
1	RDB	RNA Depletion Buffer	Yellow	-25°C to -15°C	-25°C to -15°C
1	RDE	RNA Depletion Enzyme	Yellow	-25°C to -15°C	-25°C to -15°C

Illumina cDNA Synthesis

Quantity	Reagent	Description	Cap	Shipment	Storage
4	EPH3	Elute, Prime, Fragment, High Concentration Mix	Clear	-25°C to -15°C	-25°C to -15°C
4	FSA	First Strand Synthesis Act D Mix	Amber	-25°C to -15°C	-25°C to -15°C
2	RSB	Resuspension Buffer	Clear	-25°C to -15°C	-25°C to -15°C
1	RVT	Reverse Transcriptase	Clear	-25°C to -15°C	-25°C to -15°C
4	SMM	Second Strand Marking Master Mix	Clear	-25°C to -15°C	-25°C to -15°C

Illumina RNA Prep, Ligation

Quantity	Reagent	Description	Cap Color	Shipment	Storage
4	ATL4	A-Tailing Mix	Clear	-25°C to -15°C	-25°C to -15°C
4	EPM	Enhanced PCR Mix	Clear	-25°C to -15°C	-25°C to -15°C
4	LIGX	Ligation Mix	Clear	-25°C to -15°C	-25°C to -15°C
4	RSB	Resuspension Buffer	Clear	-25°C to -15°C	-25°C to -15°C
4	STL	Stop Ligation Buffer	Red	-25°C to -15°C	-25°C to -15°C

IDT for Illumina RNA UD Indexes Set A, Ligation (96 Indexes, 96 Samples) (20040553)

Quantity	Description	Shipment	Storage
1	IDT for Illumina RNA Index Anchors	-25°C to -15°C	-25°C to -15°C
1	IDT for Illumina DNA/RNA UD Indexes Set A (UDP0001–UDP0096)	-25°C to -15°C	-25°C to -15°C

IDT for Illumina RNA UD Indexes Set B, Ligation (96 Indexes, 96 Samples) (20040554)

Quantity	Description	Shipment	Storage
1	IDT for Illumina RNA Index Anchors	-25°C to -15°C	-25°C to -15°C
1	IDT for Illumina DNA/RNA UD Indexes Set B (UDP0097–UDP0192)	-25°C to -15°C	-25°C to -15°C

IDT for Illumina RNA UD Indexes Set C, Ligation (96 Indexes, 96 Samples) (2004555)

Quantity	Description	Shipment	Storage
1	IDT for Illumina RNA Index Anchors	-25°C to -15°C	-25°C to -15°C
1	IDT for Illumina DNA/RNA UD Indexes Set C (UDP0193–UDP0288)	-25°C to -15°C	-25°C to -15°C

IDT for Illumina RNA UD Indexes Set D, Ligation (96 Indexes, 96 Samples) (20040556)

Quantity	Description	Shipment	Storage
1	IDT for Illumina RNA Index Anchors	-25°C to -15°C	-25°C to -15°C
1	IDT for Illumina DNA/RNA UD Indexes Set D (UDP0289–UDP0384)	-25°C to -15°C	-25°C to -15°C

Consumables and Equipment

The protocol has been optimized and validated using the items listed. When using alternate consumables and equipment, comparable performance is not guaranteed.

Ancillary Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes, RNase-free	General lab supplier
20 µl barrier pipette tips	General lab supplier
200 µl barrier pipette tips	General lab supplier
1000 µl barrier pipette tips	General lab supplier
96-well twin.tec 250 µl PCR plates, semiskirted	One of the following suppliers: <ul style="list-style-type: none"> • Fisher Scientific, catalog # E951020303 • VWR, catalog # 47744-106
Agencourt AMPureXP, 60 ml	Beckman Coulter, catalog # A63881
Agencourt RNAClean XP, 40 ml	Beckman Coulter, catalog # A63987
Agilent DNA 1000 Kit	Agilent Technologies, catalog # 5067-1504
Conical centrifuge tubes (15 ml or 50 ml)	General lab supplier
Ethyl alcohol, pure (500 ml)	Sigma-Aldrich, catalog # E7023
Microseal 'B' PCR Plate Sealing Film	Bio-Rad, catalog # MSB-1001
Nuclease-free ultrapure water	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, catalog # 89094-658
RNaseZap ¹	General lab supplier
[Optional] One of the following positive sample controls: <ul style="list-style-type: none"> • Human Peripheral Blood Leukocyte • Universal Human Reference RNA • Universal RNA – Mouse Normal Tissues • Universal RNA – Rat Normal Tissues • <i>E. coli</i> Total RNA 	One of the following suppliers, depending on sample control: <ul style="list-style-type: none"> • BioChain, catalog # R1234148 • Agilent Technologies, catalog # 740000 • BioChain, catalog # R4334566 • BioChain, catalog # R4334567 • Thermo Fisher Scientific, catalog # AM7940
[Optional] Agilent RNA 6000 Pico Kit	Agilent Technologies, catalog # Q32856
[Optional] Qubit Assay Tubes	Thermo Fisher Scientific, catalog # Q32856
[Optional] Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific, catalog # Q32850 or Q32853

¹ To decontaminate surfaces.

Ancillary Equipment

Equipment	Supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
2100 Bioanalyzer System	Agilent Technologies, catalog # G2939BA
[Shaking workflow] BioShake iQ high-speed thermoshaker	Q Instruments, catalog # 1808-0506
[Shaking workflow] BioShake PCR plate adapter	Q Instruments, catalog # 1808-1041

Equipment	Supplier
One of the following magnets: <ul style="list-style-type: none"> • [Shaking workflow] DynaMag-96 Side Magnet • [Pipette workflow] Magnetic Stand-96 	The applicable supplier: <ul style="list-style-type: none"> • Thermo Fisher Scientific, catalog # 12331D • Thermo Fisher Scientific, catalog # AM10027
Microplate centrifuge	General lab supplier
One of the following 96-well thermal cyclers: <ul style="list-style-type: none"> • Bio-Rad C1000 Touch Thermal Cycler • T100 Thermal Cycler 	The applicable supplier: <ul style="list-style-type: none"> • Bio-Rad, catalog # 1851196 • Bio-Rad, catalog # 1861096EDU
Vortexer	General lab supplier
[Optional] Qubit 2.0 Fluorometer	Thermo Fisher Scientific, catalog # Q32866

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
Email: techsupport@illumina.com

Illumina Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
Australia	+1.800.775.688	
Austria	+43 800006249	+43 19286540
Belgium	+32 80077160	+32 34002973
China	400.066.5835	
Denmark	+45 80820183	+45 89871156
Finland	+358 800918363	+358 974790110
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Ireland	+353 1800936608	+353 016950506
Italy	+39 800985513	+39 236003759
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Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
Taiwan	00806651752	
United Kingdom	+44 8000126019	+44 2073057197
Other countries	+44.1799.534000	

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download from support.illumina.com.



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