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TruSeq Stranded Total RNA With Illumina Ribo-Zero Plus rRNA Depletion

Reference Guide

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Revision History

Document	Date	Description of Change
Document # 1000000092426 v01	August 2019	Corrected the description of the cDNA second strand synthesis step. Corrected the Illumina catalog # for Ribo-Zero Plus rRNA Depletion (96 samples) to # 20037135.
Document # 1000000092426 v00	August 2019	Initial release.

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Chapter 1 Overview

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Introduction

This guide explains how to deplete RNA of abundant transcripts and prepare the depleted RNA for sequencing using the TruSeq™ Stranded Total RNA With Illumina® Ribo-Zero™ Plus rRNA Depletion protocol.

Reagents provided in the Ribo-Zero Plus rRNA Depletion kit remove abundant RNA using enzymatic depletion. The remaining RNA is then converted into sequencing-ready libraries for Illumina systems using TruSeq Stranded Total RNA reagents.

TruSeq Stranded Total RNA With Ribo-Zero Plus rRNA Depletion offers:

- Library capture of coding RNA and multiple forms of noncoding RNA
- Enzymatic depletion of the following abundant transcripts:
  - Human/mouse/rat cytoplasmic and mitochondrial rRNA
  - Globin transcripts
  - Bacterial rRNA
- Strand information on RNA transcripts

RNA Input Recommendations

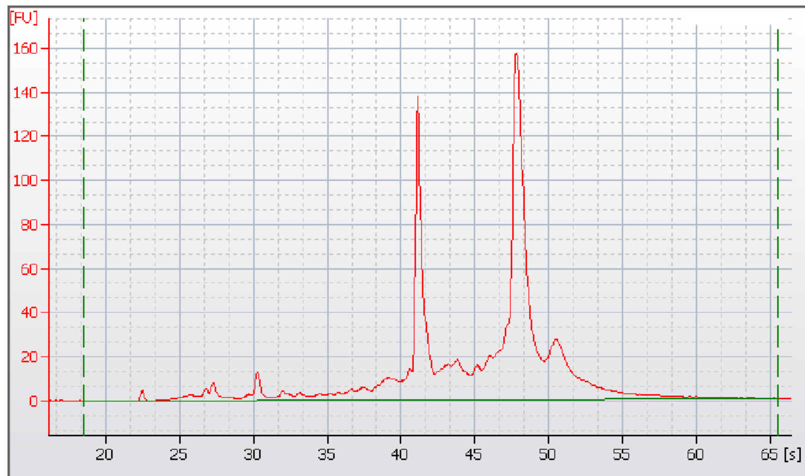
The TruSeq Stranded Total RNA With Illumina Ribo-Zero Plus rRNA Depletion protocol is optimized for 100 ng of intact RNA (RIN ≥ 7). Lower input amounts and sample quality can result in low yield. Before starting the protocol, check sample quality and quantify the total input RNA.

- Include a DNase step with the RNA isolation method to ensure purity and accurate quantification of the sample.
- This protocol has been tested for efficient rRNA depletion using the following RNA inputs:
  - Human—Universal Human Reference (UHR) RNA
  - Mouse—Universal Mouse Reference RNA
  - Rat—Universal Rat Reference RNA
  - Bacteria—Escherichia coli (Gram -) or Bacillus subtilis (Gram +) Total RNA. Performance may vary in other bacterial species.
  - Globin—Total RNA isolated from human blood.

For optimal performance, assess RNA sample quality before starting the protocol.

- The following figure shows a starting UHR Bioanalyzer trace from a RNA 6000 Pico kit.

Figure 1 Starting RNA Bioanalyzer Trace



Positive Controls

Use one of the following as positive control samples for this protocol:

- Universal Human Reference RNA (Agilent, part # 740000)
- Universal Mouse Reference RNA—Mouse Normal Tissues (BioChain, part # R4334566)
- Universal Rat Reference RNA—Rat Normal Tissues (BioChain, part # R4434567)
- E. Coli Total RNA (Thermo Fisher Scientific, part # AM7940)

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Introduction

- Before proceeding, confirm kit contents and make sure that you have the required components. This protocol requires library prep reagents, abundant RNA depletion reagents, and index adapters. See *Supporting Information* on page 20.
- Perform the protocol in the order described using specified volumes and incubation parameters.
- Avoid extended pauses in the protocol until RNA is in the form of double-stranded DNA.
- After thawing, keep all kit components on ice until required. Return reagents to storage after use.

Prepare for Pooling

If you plan to pool libraries, record information about your samples using a tool compatible with your sequencing system.

Review the planning steps in the *Index Adapter Pooling Guide (document # 1000000041074)* and the *Library Pooling Guidelines for the NextSeq and MiniSeq Systems* technical bulletin when preparing libraries for Illumina sequencing systems that require balanced index combinations.

Tips and Techniques

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 index adapters with a multichannel pipette, change tips between ***each row*** or ***each column***.

Handling RNA

- Always set aside RNA sample plate on ice or on a cooling block.
- When not in use, seal plates and close lids to limit contamination.
- Avoid multiple freeze-thaw cycle 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 into separate tubes for single-use.

Sealing the Plate

- Always seal the 96-well plate before the following steps in the protocol:
  - Centrifuging steps
  - Thermal cycling steps
- Apply the adhesive seal to cover the plate, and seal with a rubber roller.
- Microseal 'B' adhesive seals are effective at -40°C to 110°C and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' for thermal cycling steps and short-term storage.

Plate Transfers

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

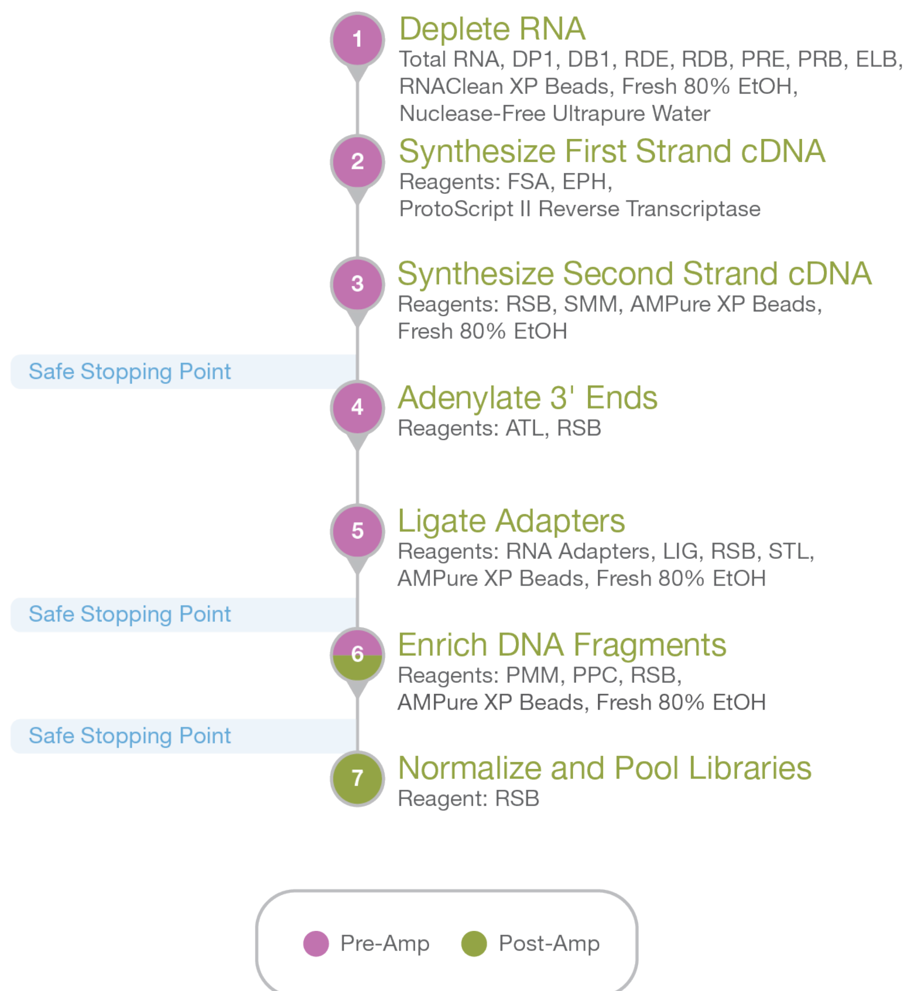
Handling Beads

- Do not freeze beads.
- Pipette bead suspensions slowly.
- Before use, allow the beads to come to room temperature.
- Immediately before use, vortex the beads until they are well dispersed. The color of the liquid must appear homogeneous. Vortex throughout protocol as necessary to keep homogeneous.
- If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the liquid is clear (1–2 minutes).
- When washing beads:
  - Use the specified magnetic stand for the plate.
  - Dispense liquid so that beads on the side of the wells are wetted.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - Do not agitate the plate while it is on the magnetic stand. Do not disturb the bead pellet.
  - Do not over-dry beads.

Library Prep Workflow

The following diagram illustrates the TruSeq Stranded Total RNA With Illumina Ribo-Zero Plus rRNA Depletion workflow. Safe stopping points are marked between steps.

Figure 2 TruSeq Stranded Total RNA With Illumina Ribo-Zero Plus rRNA Depletion Workflow



Deplete rRNA

This step removes abundant RNA from purified total RNA using the Ribo-Zero Plus rRNA Depletion kit.

Consumables

- DB1 (Depletion Probe Buffer)
- DP1 (Depletion Probe Pool)
- ELB (Elution Buffer)
- PRB (Probe Removal Buffer)
- PRE (Probe Removal Enzyme)

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- RDB (RNA Depletion Buffer)
- RDE (RNA Depletion Enzyme)
- Freshly prepared 80% ethanol (EtOH)
- Nuclease-free ultrapure water
- Agencourt RNAClean XP beads
- 96-well 0.25 ml PCR plates, semiskirted
- Microseal 'B' adhesive seals
- RNase-free 1.7 ml microcentrifuge tubes
- Prepare for later procedure:
    - EPH (Elute, Prime, Fragment High Mix)
    - FSA (First Strand Synthesis Act D Mix)

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
Agencourt RNAClean XP beads	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
ELB	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
DB1 (blue cap)	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
DP1 (blue cap)	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
EPH	-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. Invert to mix, and then centrifuge briefly.
PRB (red cap)	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
PRE (red cap)	-25°C to -15°C	Keep in storage until required. Flick tube to mix, and then centrifuge briefly.
RDB (yellow cap)	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
RDE (yellow cap)	-25°C to -15°C	Keep in storage until required. Flick tube to mix, then centrifuge briefly.

2 Save the following HYB_DP1 program on the thermal cycler:
- Choose the preheat lid option and set to 105°C
- 95°C for 2 minutes
- Decrease 0.1°C per second until temperature reaches 37°C
- Hold at 37°C

3 Save the following RNA_DEP program on the thermal cycler:
- Choose the preheat lid option and set to 105°C
- 37°C for 15 minutes
- Hold at 4°C

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4 Save the following PRB_REM program on the thermal cycler:
   - Choose the preheat lid option and set to 105°C
   - 37°C for 15 minutes
   - Hold at 4°C

Procedure

Hybridize Probes

1 Dilute 100 ng total RNA in 11 µl using nuclease-free ultrapure water in each well of a 96-well PCR plate.

2 Combine the following volumes in a 1.7 ml tube on ice to prepare a Hybridize Probe Master Mix. For multiple samples, multiply each volume by the number of samples.
   - DP1 (1.2 µl) (blue cap)
   - DB1 (3.6 µl) (blue cap)

   These volumes produce 4 µl Hybridize Probe Master Mix per well. Reagent overage is included in volumes to ensure accurate pipetting.

3 Pipette thoroughly to mix.

4 Add 4 µl master mix to each well.

5 Pipette up and down 10 times to mix, and then seal.

6 [Optional] Centrifuge at 280 x g for 10 seconds.

7 Place on the preprogrammed thermal cycler and run the HYB_DP1 program.
   Each well contains 15 µl sample.

Deplete rRNA

1 Combine the following volumes in a 1.7 ml tube on ice to prepare a rRNA Depletion Master Mix. For multiple samples, multiply each volume by the number of samples.
   - RDE (1.2 µl) (yellow cap)
   - RDB (4.8 µl) (yellow cap)

   These volumes produce 5 µl rRNA Depletion Master Mix per well. Reagent overage is included in volumes to ensure accurate pipetting.

2 Pipette thoroughly to mix.

3 Centrifuge the sealed sample plate at 280 x g for 10 seconds.

4 Add 5 µl master mix to each well.

5 Pipette up and down 10 times to mix, and then seal.

6 [Optional] Centrifuge at 280 x g for 10 seconds.

7 Place on the preprogrammed thermal cycler and run the RNA_DEP program.
   Each well contains 20 µl sample.

Remove Probes

1 Combine the following volumes in a 1.7 ml tube on ice to prepare a Probe Removal Master Mix. For multiple samples, multiply each volume by the number of samples.
   - PRE (3.3 µl) (red cap)
   - PRB (7.7 µl) (red cap)

These volumes produce 10 µl Probe Removal Master Mix per well. Reagent overage is included in volumes to ensure accurate pipetting

2 Pipette thoroughly to mix.
3 Centrifuge the sealed sample plate at 280 x g for 10 seconds.
4 Add 10 µl master mix to each well.
5 Pipette up and down 10 times to mix, and then seal.
6 **[Optional]** Centrifuge at 280 x g for 10 seconds.
7 Place on the preprogrammed thermal cycler and run the PRB_REM program.
Each well contains 30 µl sample.

Clean Up RNA

1. Centrifuge the sealed sample plate at 280 x g for 10 seconds.
2. Add 60 µl RNAClean XP beads to each well.
3. Pipette up and down until beads are fully resuspended.
4. Incubate at room temperature for 5 minutes.
5. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
6. Remove and discard all supernatant from each well.
7. Wash beads as follows.
   a. Keep on magnetic stand and add 175 µl fresh 80% ethanol to each well.
   b. Wait 30 seconds.
   c. Remove and discard all supernatant from each well.
8. Wash beads a **second** time.
9. Remove all residual ethanol from each well.
10. Air-dry on the magnetic stand for 1 minute.
11. Remove from the magnetic stand.
12. Add 10.5 µl ELB onto beads in each well.
13. Slowly pipette to mix until beads are fully resuspended.
14. Incubate at room temperature for 2 minutes.
15. Seal and then centrifuge at 280 x g for 10 seconds.
16. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
17. Transfer 8.5 µl supernatant to the corresponding well of a new plate.

Fragment and Denature RNA

This step fragments and primes the depleted RNA using random hexamers for cDNA synthesis.

Consumables

- EPH (Elute, Prime, Fragment High Mix)

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Preparation

1 Save the following DEN_RNA program on the thermal cycler:
   - Choose the preheat lid option and set to 105°C
   - 94°C for 2 minutes
   - Hold at 4°C

Procedure

1 Add 8.5 µl EPH to each well.

2 Pipette up and down 10 times to mix, and seal.

3 [Optional] Centrifuge at 280 x g for 10 seconds.

4 Place on the preprogrammed thermal cycler and run the DEN_RNA program.
Each well contains 17 µl sample.

Synthesize First Strand cDNA

This step reverse transcribes the cleaved RNA fragments primed with random hexamers into first strand cDNA. The addition of Actinomycin D to the FSA (First Strand Synthesis Act D Mix) prevents spurious DNA-dependent synthesis, while allowing RNA-dependent synthesis and improving strand specificity.

Consumables

- FSA (First Strand Synthesis Act D Mix)
- ProtoScript II Reverse Transcriptase
- Microseal 'B' adhesive seals
- RNase-free 1.7 ml microcentrifuge tubes
- Prepare for later procedure:
   - RSB (Resuspension Buffer)
   - SMM (Second Strand Marking Master Mix)
   - Agencourt AMPure XP beads

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. See the safety data sheet (SDS) for environmental, health, and safety information. For more information, see *Technical Assistance* on page 24.

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
ProtoScript II	-25°C to -15°C	Keep in storage until required. Flick tube to mix, and then centrifuge briefly.
SMM	-25°C to -15°C	Thaw on ice. Invert to mix, and then centrifuge briefly.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
AMPure XP beads	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.

2 Save the following FSS program on the thermal cycler:
   - Choose the preheat lid option and set to 105°C
   - 25°C for 10 minutes
   - 42°C for 15 minutes
   - 70°C for 15 minutes
   - Hold at 4°C

Procedure

1 Combine the following volumes in a 1.7 ml tube on ice to prepare the First Strand Synthesis master mix. For multiple samples, multiply each volume by the number of samples.
   - FSA (9 µl)
   - ProtoScript II (1 µl)

   These volumes produce 8 µl First Strand Synthesis master mix per well. Reagent overage is included in volumes to ensure accurate pipetting.

2 Pipette thoroughly to mix.

3 Centrifuge the sealed sample plate at 280 x g for 10 seconds.

4 Add 8 µl master mix to each well.

5 Pipette up and down 10 times to mix, and then seal.

6 [Optional] Centrifuge at 280 x g for 10 seconds.

7 Place on the preprogrammed thermal cycler and run the FSS program.
   Each well contains 25 µl.

Synthesize Second Strand cDNA

This step removes the RNA template and synthesizes a replacement strand to generate double-stranded cDNA. dUTP is incorporated in place of dTTP to quench the second strand during amplification. Magnetic beads separate the double-stranded cDNA from the Second Strand Marking Master Mix.

Consumables

- RSB (Resuspension Buffer)
- SMM (Second Strand Marking Master Mix)
- Agencourt AMPure XP beads
- Freshly prepared 80% ethanol (EtOH)
- 96-well 0.25 ml PCR plate, semiskirted
- Microseal 'B' adhesive seals

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- RNase-free 1.7 ml microcentrifuge tubes

About Reagents

- Vortex AMPure XP beads before each use.
- Vortex AMPure XP beads frequently to make sure that beads are evenly distributed.
- Aspirate and dispense AMPure XP beads slowly due to the viscosity of the solution.

Preparation

1. Save the following SSS program on the thermal cycler:
   - Choose the preheat lid option and set to 40°C
   - 16°C for 1 hour
   - Hold at 4°C

Procedure

Add SMM

1. Combine the following volumes in a 1.7 ml tube on ice to prepare Second Strand Synthesis master mix. For multiple samples, multiply each volume by the number of samples.
   - RSB (5.3 µl)
   - SMM (21.2 µl)

   These volumes produce 25 µl Second Strand Synthesis master mix per well. Reagent overage is included in volumes to ensure accurate pipetting.
2. Pipette thoroughly to mix.
3. Centrifuge the sealed sample plate at 280 x g for 10 seconds.
4. Add 25 µl master mix to each well.
5. Pipette up and down 5 times to mix, and then seal.
6. [Optional] Centrifuge at 280 x g for 10 seconds.
7. Place on the preprogrammed thermal cycler and run the SSS program.
   Each well contains 50 µl.

Purify cDNA

1. Centrifuge the sealed sample plate at 280 x g for 10 seconds.
2. Add 90 µl AMPure XP beads to each well.
3. Pipette up and down until beads are fully resuspended.
4. Incubate at room temperature for 5 minutes.
5. Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
6. Remove and discard all supernatant from each well.
7. Wash beads as follows.
   a. Keep on magnetic stand and add 175 µl fresh 80% ethanol to each well.
   b. Wait 30 seconds.

   c Remove and discard all supernatant from each well.

8 Wash beads a **second** time.

9 Remove residual ethanol from each well.

10 Air-dry on the magnetic stand for 1 minute. Do not over dry beads.

11 Remove from the magnetic stand.

12 Add 19.5 µl RSB directly onto the beads in each well.

13 Slowly pipette to mix until beads are fully resuspended.

14 Incubate at room temperature for 2 minutes.

15 Seal and then centrifuge at 280 × g for 10 seconds.

16 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

17 Transfer 17.5 µl supernatant to the corresponding well of a new plate.

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 one adenine (A) nucleotide to the 3′ ends of the blunt fragments to prevent them from ligating to each other during adapter ligation reaction. One corresponding thymine (T) nucleotide on the 3′ end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

- ATL (A-Tailing Mix)
- Microseal 'B' adhesive seals
- 1.7 ml microcentrifuge tubes
- Prepare for later procedure:
  - Index adapter plate
  - STL (Stop Ligation Buffer)

Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
ATL	-25°C to -15°C	Thaw on ice. 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 x g for 1 minute.
STL	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.

2 Save the following ATAIL program on the thermal cycler:
- Choose the preheat lid option and set to 105°C
- 37°C for 30 minutes
- 70°C for 5 minutes

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- Hold at 4°C

Procedure

1. Centrifuge the sealed sample plate at 280 x g for 10 seconds.
2. Add 12.5 µl ATL to each well.
3. Pipette up and down 10 times to mix, and then seal.
4. [Optional] Centrifuge at 280 x g for 10 seconds.
5. Place on the preprogrammed thermal cycler and run the ATAIL program.
   Each well contains 30 µl.

Ligate Adapters

This step ligates multiple indexing adapters to the ends of the double-stranded cDNA fragments, which prepares them for hybridization onto a flow cell.

Index adapters must be ordered separately from the library prep components. For information on compatible index adapters, see *Supporting Information* on page 20.

Consumables

- LIG (Ligation Mix)
- Index adapter plate
- STL (Stop Ligation Buffer)
- 96-well 0.25 ml PCR Plate, semiskirted
- Microseal 'B' adhesive seals
- 1.7 ml microcentrifuge tubes
- Prepare for later procedure:
  - Agencourt AMPure XP beads
  - RSB (Resuspension Buffer)

About Reagents

- Return LIG to storage immediately after use.
- The index plate wells cannot be reused.

Preparation

1. Prepare the following consumables:

Reagent	Storage	Instructions
LIG	-25°C to -15°C	Keep in storage until required. Flick tube to mix, and then centrifuge briefly.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.
AMPure XP beads	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix.

2. Pierce the foil seal on the index adapter plate with a new pipette tip for each well for only the number of

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samples being processed.

3 Save the following LIG program on the thermal cycler:
   - Choose the preheat lid option and set to 105°C
   - 30°C for 10 minutes
   - Hold at 4°C

Procedure

Add Index Adapters

1 Centrifuge the sealed sample plate at 280 x g for 10 seconds.

2 Add the following volumes to each sample well ***in the order listed***. Do not combine to make a master mix.
   - RSB (2.5 µl)
   - LIG (2.5 µl)
   - Index adapters (2.5 µl)

3 Pipette thoroughly to mix, and then seal.

4 [Optional] Centrifuge at 280 × g for 10 seconds.

5 Place on the preprogrammed thermal cycler and run the LIG program.
   Each well contains 37.5 µl.

Stop Ligation

1 Centrifuge the sealed sample plate at 280 x g for 10 seconds.

2 Add 5 µl STL to each well.

3 Pipette up and down 15 times to mix.

4 [Optional] Seal and then centrifuge at 280 x g for 10 seconds.

Clean Up Libraries

This step uses Agencourt AMPure XP beads to clean up the library.

Consumables

- Agencourt AMPure XP beads
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)

About Reagents

- Vortex AMPure XP beads before each use.
- Vortex AMPure XP beads frequently to make sure that beads are evenly distributed.
- Aspirate and dispense AMPure XP beads slowly due to the viscosity of the solution.

Procedure

1 Use the round 1 volumes indicated in the following table for steps 2–17.

Component	Round 1	Round 2
AMPure XP Beads	34 µl	40 µl
RSB	52 µl	22 µl
Supernatant to transfer	50 µl	20 µl

2 Add AMPure XP beads to each well.

3 Pipette up and down until beads are fully resuspended.

4 Incubate at room temperature for 5 minutes.

5 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

6 Remove and discard all supernatant from each well.

7 Wash beads as follows.

   a Keep on magnetic stand and add 175 µl fresh 80% ethanol to each well.
   b Wait 30 seconds.
   c Remove and discard all supernatant from each well.

8 Wash beads a **second** time.

9 Remove residual ethanol from each well.

10 Air-dry on the magnetic stand for 1 minute. Do not over dry beads.

11 Remove from the magnetic stand.

12 Add RSB directly onto beads in each well.

13 Slowly pipette up and down until beads are fully resuspended.

14 Incubate at room temperature for 2 minutes.

15 Seal and then centrifuge at 280 x g for 10 seconds.

16 Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

17 Transfer supernatant to the corresponding well of a new plate. Use the supernatant volume indicated in the table above.

18 Repeat steps 2–17 using the round 2 volumes.

Amplify DNA Fragments

This step uses PCR to selectively amplify DNA fragments with adapter molecules on both ends. PCR is performed with PPC (PCR Primer Cocktail) that anneals to the ends of the adapters.

Consumables

- PMM (PCR Master Mix)
- PPC (PCR Primer Cocktail)
- 96-well 0.25 ml PCR Plate, semiskirted
- Microseal 'B' adhesive seals
- 1.7 ml microcentrifuge tubes

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Preparation

1 Prepare the following consumables:

Reagent	Storage	Instructions
PPC	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
PMM	-25°C to -15°C	Thaw on ice. Invert to mix, and then centrifuge briefly.

2 Save the following PCR program on the thermal cycler:
- Choose the preheat lid option and set to 105°C
- 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

Sample Type	Number of PCR Cycles (X)
Bacteria	15
All other sample types	13

Procedure

1 Combine the following volumes in a 1.7 ml tube on ice to prepare the PCR reaction mix. For multiple samples, multiply each volume by the number of samples.
- PPC (5.2 µl)
- PMM (26 µl)

These volumes produce 30 µl PCR reaction mix per well. Reagent overage is included to ensure accurate pipetting.

2 Pipette thoroughly to mix.

3 Seal and then centrifuge at 280 x g for 10 seconds.

4 Add 30 µl PCR reaction mix to each well.

5 Pipette up and down 5 times to mix, and then seal.

6 [Optional] Centrifuge at 280 x g for 10 seconds.

7 Place on the preprogrammed thermal cycler and run the PCR program.
Each well contains 50 µl.

SAFE STOPPING POINT

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

Perform Second Cleanup

This step uses Agencourt AMPure XP beads to perform a second cleanup.

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Consumables

- Agencourt AMPure XP beads
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)

About Reagents

- Vortex AMPure XP beads before each use.
- Vortex AMPure XP beads frequently to make sure that beads are evenly distributed.
- Aspirate and dispense AMPure XP beads slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables:

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

Procedure

1 Centrifuge the sealed sample plate at 280 × g for 10 seconds.

2 Add 50 µl AMPure XP beads to each well.

3 Pipette up and down until beads are fully resuspended.

4 Incubate at room temperature for 5 minutes.

5 Place on a magnetic stand and wait until the liquid is clear (~5 minutes).

6 Remove and discard all supernatant from each well.

7 Wash beads as follows.

   a Keep on magnetic stand and add 175 µl fresh 80% ethanol to each well.
   b Wait 30 seconds.
   c Remove and discard all supernatant from each well.

8 Wash beads a **second** time.

9 Remove residual ethanol from each well.

10 Air-dry on the magnetic stand for 1 minute.

11 Remove from the magnetic stand.

12 Add 22 µl RSB directly onto the beads in each well.

13 Slowly pipette to mix until beads are fully resuspended.

14 Incubate at room temperature for 2 minutes.

15 Seal and then centrifuge at 280 × g for 10 seconds.

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16 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

17 Transfer 20 µl supernatant to the corresponding well of a new plate.

SAFE STOPPING POINT

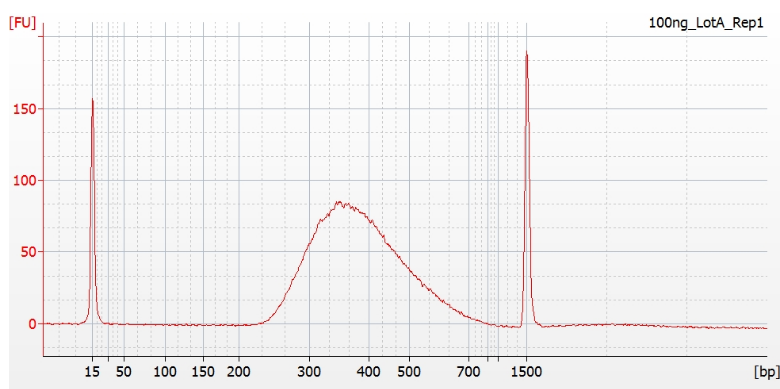
If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Check Libraries

Perform the following procedure to check the final library concentration and quality.

1 Analyze 1 µl pooled library or individual library on the Agilent 2100 Bioanalyzer using a DNA 1000 kit.

Figure 3 Example Bioanalyzer trace



2 [Optional] Analyze 2 µl library using the Qubit dsDNA BR Assay Kit for further quantification.
Intact RNA samples result in an average library fragment length of ~350–400 bp. The expected insert size is ~200 bp.

Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

For sequencing, Illumina recommends setting up a paired-end run of 76 cycles per read (2 × 76 run format) and 8 cycles per index read.

1 Obtain the molarity value of the library or pooled libraries using one of the following methods:
- For libraries quantified on a Bioanalyzer, use the molarity value obtained for the library.
- For libraries quantified on a Qubit, calculate the molarity value using the following formula. Use the average size obtained from the Bioanalyzer and the concentration obtained from the Qubit.

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

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	4	0.8–1

3 Dilute libraries using RSB:

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- **Libraries quantified as a pool**—Dilute the pool to the starting concentration for your system.
- **Libraries quantified individually**—Dilute each library to the starting concentration for your system. Add 10 µl each diluted library to a tube to create a pool.

4 Follow the denature and dilute instructions for your system to dilute to the final loading concentration. Use the final loading concentration indicated in the table above.

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Supporting Information

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Introduction

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

Kit Contents

Completing the TruSeq Stranded Total RNA With Ribo-Zero Plus rRNA Depletion protocol requires depletion reagents, library prep reagents, and index adapters.

Component	Kit	Catalog #
Depletion Kit	Ribo-Zero Plus rRNA Depletion (96 samples)	20037135
Library Prep Kit	TruSeq Stranded Total RNA Human/Mouse/Rat (96 Samples)	20020597
Indexes	IDT for Illumina TruSeq RNA UD Indexes (24 indexes, 96 samples)	20020591
	IDT for Illumina TruSeq RNA UD Indexes (96 indexes, 96 samples)	20022371

Ribo-Zero Plus rRNA Depletion Kit, Store at -25°C to -15°C

This depletion protocol uses the components described in the following table. One box is included for the 96 sample workflow.

NOTE

Agencourt RNAClean XP beads are not included in this kit and must be purchased separately.

Quantity	Reagent	Description
1	DP1	Depletion Probe Pool
1	DB1	Depletion Probe Buffer
1	RDE	RNA Depletion Enzyme
1	RDB	RNA Depletion Buffer
1	PRE	Probe Removal Enzyme
1	PRB	Probe Removal Buffer
1	ELB	Elution Buffer

TruSeq Stranded Total RNA Library Prep (96 Samples)

This library prep protocol uses the components described in the sections that follow. The TruSeq Stranded Library Prep (96 Samples) kit contains additional components that are not required for the TruSeq Stranded Total RNA With Ribo-Zero Plus rRNA Depletion protocol. A quantity of two of each box is included for the 96 sample workflow.

NOTE

ProtoScript II Reverse Transcriptase and AMPure XP beads are not included in this kit and must be purchased separately.

Core Library Prep Box 1, Store at -25°C to -15°C

Quantity	Reagent	Description
1	ATL	A-Tailing Mix
1	CTA	A-Tailing Control
1	CTE	End Repair Control
1	CTL	Ligation Control
1	LIG	Ligation Mix
1	RSB	Resuspension Buffer
1	STL	Stop Ligation Buffer

Core Library Prep Box 2, Store at -25°C to -15°C

Quantity	Reagent	Description
1	PMM	PCR Master Mix
1	PPC	PCR Primer Cocktail
1	FSA	First Strand Synthesis Act D Mix
1	SMM	Second Strand Marking Master Mix

Library Prep Box 1, Store as Specified

Quantity	Reagent	Description	Storage Temperature
2	RRB	rRNA Removal Beads	2°C to 8°C
1	DTL	CTL Dilution Tube	Room temperature
1	DTE	CTE Dilution Tube	Room temperature
1	DTA	CTA Dilution Tube	Room temperature

IDT for Illumina TruSeq RNA UD Indexes, Store at -25°C to 15°C

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

Quantity	Description
1	IDT for Illumina TruSeq RNA UD Indexes (24 Indexes, 96 Samples) or IDT for Illumina TruSeq RNA UD Indexes (96 Indexes, 96 Samples)

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.

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Consumables

Consumable	Supplier
1.7 ml RNase/DNase-free tubes	General lab supplier
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
96-well 250 µl PCR plates	Bio-Rad, part # 12001925 or Eppendorf, part # 951020303
Agencourt AMPureXP 60 ml kit	Beckman Coulter Genomics, part # A63881
Agencourt RNAClean XP 40 ml kit	Beckman Coulter Genomics, part # A63987
Agilent DNA 1000 Kit	Agilent Technologies, part # 5067-1504
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
Nuclease-free ultrapure water	General lab supplier
Protoscript II Reverse Transcriptase	NEB, part # M0368L
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
RNaseZap (to decontaminate surfaces)	General lab supplier
[Optional] Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific, part # Q32853
[Optional] One of the following positive sample controls: • Universal Human Reference RNA • Universal RNA – Mouse Normal Tissues • Universal RNA – Rat Normal Tissues • E.Coli Total RNA	One of the following suppliers, depending on sample control: • Agilent Technologies, part # 740000 • BioChain, part # R4334566 • BioChain, part # R4434567 • Thermo Fisher Scientific, part # AM7940
[Optional] Agilent RNA 6000 Pico Kit	• Agilent Technologies, part # 5067-1513

Equipment

Equipment	Supplier
96-well thermal cycler (with programmable heated lid)	Bio-Rad, part # 1861096
2100 Bioanalyzer Instrument	Agilent Technologies, part # G2939BA
[Optional] Qubit Fluorometer	Thermo Fisher Scientific, part # Q32866
Magnetic stand-96	Thermo Fisher Scientific, part # AM10027
Microplate centrifuge	General lab supplier
Vortexer	General lab supplier

Acronyms

Acronym	Definition
ATL	A-Tailing Mix
DB1	Depletion Probe Buffer
DP1	Depletion Probe Pool
ELB	Elution Buffer
EPH	Elute, Prime, Fragment High Mix
FSA	First Strand Synthesis Act D Mix
IEM	Illumina Experiment Manager
LIG	Ligation Mix
LRM	Local Run Manager
PCR	Polymerase Chain Reaction
PMM	PCR Master Mix
PPC	PCR Primer Cocktail
PRB	Probe Removal Buffer
RDB	RNA Depletion Buffer
RDE	RNA Depletion Enzyme
RSB	Resuspension Buffer
SMM	Second Strand Marking Master Mix
STL	Stop Ligation Buffer

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Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
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Illumina Customer Support Telephone Numbers

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Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
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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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