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Revision History

Part #	Revision	Date	Description of Change
15031048	E	October 2013	<ul style="list-style-type: none"> • Clarified PDP plate type options for LS protocol are a 0.3 ml PCR plate when pooling \leq 40 samples or a 96-well MIDI plate when pooling $>$ 40 samples • Created new appendix of <i>Supporting Information</i> containing <i>Acronyms, Kit Contents, Consumables and Equipment, and Indexed Adapter Sequences</i> • Replaced <i>Best Practices</i> section with a reference to content on the Illumina website • Replaced <i>Adapter Options</i> and <i>Pooling Guidelines</i> sections with a reference to the <i>TruSeq Sample Preparation Pooling Guide (part # 15042173)</i>
15031048	D	April 2013	<ul style="list-style-type: none"> • Added new TruSeq Stranded Total RNA plant and globin kit information in the following sections. <ul style="list-style-type: none"> • <i>Introduction</i> • <i>Acronyms</i> • <i>Kit Contents</i> • <i>Ribo-Zero Deplete and Fragment RNA</i> procedures • <i>Usage Guidelines</i> • Added bioanalyzer and DNA 1000 Kit to equipment list. • Corrected <i>Kit Contents</i> box 1 shipping temperature. • Corrected storage temperature of rRNA Binding Buffer to -15°C to -25°C. • Removed the SIP and RIP plate transfer steps from the <i>Ribo-Zero Deplete and Fragment RNA</i> procedures • In <i>Clean Up PCR HS</i> protocol, added centrifuge step to mixing procedures to make mixing consistent throughout protocol • Clarified in protocol steps that the PDP plate is a 0.3 ml PCR plate in the LS protocol and an HSP plate in the HS protocol • In the <i>Alternate Fragmentation Protocols</i> Appendix, added an elution step for intact RNA with an insert length of 130–350 bp • Moved Usage Guidelines to an Appendix

Part #	Revision	Date	Description of Change
15031048	C	September 2012	<ul style="list-style-type: none"> • Added New England Biolabs, Inc. licensing to notices • Corrected PCR Primer Cocktail part number in LT Kit Contents • Corrected kit name with 96 Sample, cDNA Synthesis-PCR Box • Reformatted the consumables list at the start of each procedure to a table • After initial thaw, for each process that uses Resuspension Buffer, added a preparation step to remove it from 2°C to 8°C storage
15031048	B	July 2012	<ul style="list-style-type: none"> • Added TruSeq Stranded Total RNA HT Sample Prep Kit content and functionality to the following sections: <ul style="list-style-type: none"> • <i>Usage Guidelines</i> • <i>Kit Contents</i> • <i>Indexed Adapter Sequences</i> • <i>Adapter Options</i> • <i>Pooling Guidelines</i> • <i>Ligate Adapters</i> procedures • <i>Enrich DNA Fragments</i> procedures • <i>Normalize and Pool Libraries</i> procedures • Added reagent volume table to <i>Usage Guidelines</i> • Revised <i>Tracking Tools</i> documentation download information • Removed detailed Sample Sheet description from <i>Tracking Tools</i> • Added instructions for which assay to select when using the Illumina Experiment Manager • Corrected storage temperature for rRNA Binding Buffer and Elution Buffer as 2° to 8°C • Added optional Agilent RNA 6000 Nano or Pico kits for alternative fragmentation to <i>Consumables and Equipment</i> list • Specified storage temperature for Resuspension Buffer at 2° to 8°C after initial thaw • Make RRP - Added steps to transfer supernatant from RIP to SIP plate and incubate
15031048	A	April 2012	Initial Release

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Introduction

This protocol explains how to convert total RNA into a library of template molecules of known strand origin and suitable for subsequent cluster generation and DNA sequencing using the reagents provided in the Illumina® TruSeq® Stranded Total RNA Sample Preparation kits. TruSeq Stranded Total RNA with Ribo-Zero™ Human/Mouse/Rat, TruSeq Stranded Total RNA with Ribo-Zero Gold, and TruSeq Stranded Total RNA with Ribo-Zero Globin support human, mouse, and rat organisms, whereas TruSeq Stranded Total RNA with Ribo-Zero Plant supports plant species.

All TruSeq Stranded Total RNA kits follow the same workflow. The first step involves the removal of ribosomal RNA (rRNA) using biotinylated, target-specific oligos combined with Ribo-Zero rRNA removal beads. The Ribo-Zero Human/Mouse/Rat kit depletes samples of cytoplasmic rRNA and the Ribo-Zero Gold kit depletes samples of both cytoplasmic and mitochondrial rRNA. Ribo-Zero Globin kit depletes globin-encoding mRNA in addition to the rRNA species targeted with Ribo-Zero Gold. Ribo-Zero Plant kit targets cytoplasmic and chloroplast rRNA. Following purification, the RNA is fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments are copied into first strand cDNA using reverse transcriptase and random primers, followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. These cDNA fragments then have the addition of a single 'A' base and subsequent ligation of the adapter. The products are purified and enriched with PCR to create the final cDNA library.

This sample preparation protocol offers:

- ▶ Strand information on RNA transcript
- ▶ Library capture of both coding RNA, as well as multiple forms of non-coding RNA
- ▶ Degraded RNA can be used with minor adjustments to fragmentation procedures
- ▶ Reduced total assay time
- ▶ Optimized workflows for processing low sample (LS) and high sample (HS) numbers in parallel
- ▶ Compatibility with low-throughput (LT) and high-throughput (HT) kit configurations
- ▶ The TruSeq Stranded Total RNA LT Sample Prep Kit contains adapter index tubes recommended for preparing and pooling 24 or fewer samples for sequencing
- ▶ The TruSeq Stranded Total RNA HT Sample Prep Kit contains a 96-well plate with 96 uniquely indexed adapter combinations designed for manual or automated preparation of 96 uniquely indexed samples

The protocol is compatible with no indexing or a lower indexing pooling level. The libraries generated do not require PCR amplification to enable cluster generation, although

PCR is recommended in the standard protocol to robustly meet the yield requirements of most standard applications.

Protocol Features

This guide documents the sample preparation protocol using the Illumina TruSeq Stranded Total RNA LT Sample Prep Kit or TruSeq Stranded Total RNA HT Sample Prep Kit.

- ▶ Chapter 2 Low Sample (LS) Protocol explains how to perform the TruSeq Stranded Total RNA Sample Preparation using the Low Sample Protocol
- ▶ Chapter 3 High Sample (HS) Protocol explains how to perform the TruSeq Stranded Total RNA Sample Preparation using the High Sample Protocol

Equivalent results can be expected from either protocol and their distinguishing elements are as follows:

Table 1 Protocol Features

	Low Sample	High Sample
LT Kit - Number of samples processed at one time	≤ 48 with indexed adapter tubes	> 48 with indexed adapter tubes
HT Kit - Number of samples processed at one time	≤ 24 with indexed adapter plate	> 24 with indexed adapter plate
Plate Type	96-well 0.3 ml PCR 96-well MIDI	96-well HSP 96-well MIDI
Incubation Equipment	96-well thermal cycler	96-well thermal cycler Microheating system
Mixing Method	Pipetting	Microplate shaker

Illumina recommends the following kit, sample number, and protocol combinations:

Table 2 Kit and Sample Number Recommendations

Number of Samples Processed At One Time	Recommended Kit
<24	LT
24–48	LT or HT
>48	HT

Table 3 Kit and Protocol Recommendations

Kit	Number of Samples Supported	Number of Samples Processed At One Time	Protocol
LT	48	≤48	LS
		>48	HS
HT	96	≤24	LS
		>24	HS

RNA Input Recommendations

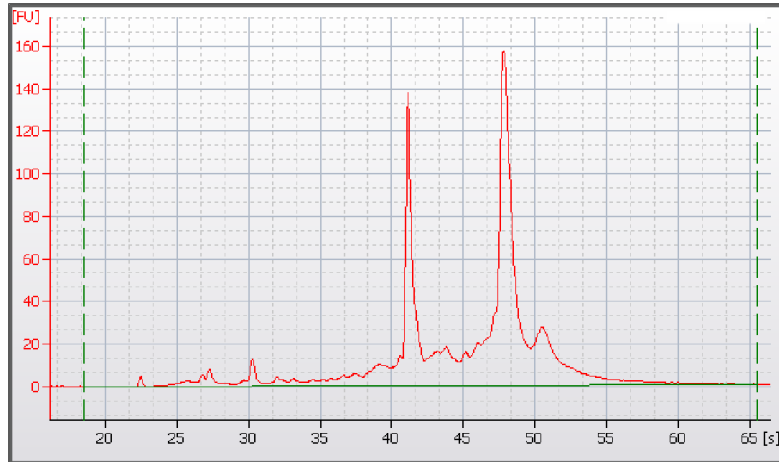
It is important to follow the TruSeq Stranded Total RNA Sample Preparation input recommendations.

Total RNA Input

- ▶ This protocol is optimized for 0.1–1 µg of total RNA.
 - Lower amounts might result in inefficient ligation and low yield.
- ▶ The protocol has been tested using 0.1–1 µg of high-quality universal human reference total RNA as input.
 - Use of RNA from other species, tissues, or qualities might require further optimization regarding the initial input amount.
- ▶ The protocol recommends diluting the in-line controls for tracking the steps involved in converting dsDNA into libraries.
 - The dilution is optimized for 0.1–1 µg of high-quality input RNA.
 - When using less RNA or highly degraded RNA, these controls might need further dilution.
 - If no controls are added, use Resuspension Buffer in place of the controls in the protocol.
- ▶ It is important to know the quality of the RNA starting material. The fragmentation conditions were optimized for high-quality RNA.
 - Degraded RNAs are shorter than full length RNA. If the same fragmentation conditions for degraded RNAs are used, it will cause the libraries to be shorter and can result in low yield or failure of the protocol.
 - If starting with degraded RNA, the fragmentation time must be adjusted to avoid over fragmentation of the RNA samples. For more information, see Appendix A Alternate Fragmentation Protocols.
 - RNA that has DNA contamination will result in an underestimation of the amount of RNA used.

- ▶ The following figure shows a Universal Human Reference (UHR) starting RNA Bioanalyzer trace.

Figure 1 Starting RNA Bioanalyzer Trace



Positive Control

Illumina recommends using Agilent Technologies Human UHR total RNA (catalog # 740000) as a positive control sample for this protocol.

In-Line Control DNA

The End Repair Control, A-Tailing Control, and Ligation Control reagents contain DNA fragments used as controls for the enzymatic activities of the Second Strand Marking Master Mix, A-Tailing Mix, and Ligation Mix, respectively. Each reagent contains dsDNA fragments designed to report the success or failure of a specific enzymatic activity used in the library preparation process. Sequencing determines the readout. If the sequence of an in-line control is in the final sequencing data viewed in the Sequence Analysis Viewer (SAV), it indicates that its corresponding step was successful. If it does not, or if it is in substantially diminished numbers, it indicates the step failed. The controls are intended for troubleshooting and are useful for identifying the specific mode of failure, but are uninformative in cases where sequencing data are not generated from a library.



NOTE

The use of these controls is optional and they can be replaced with the same volume of Resuspension Buffer.

The control molecules work through the design of their ends. Controls are added to the reactions before their corresponding step in the protocol. Their end structures match those of a DNA molecule that has not gone through the step. If the step is successful, the control molecule will be modified to participate in downstream reactions of library generation and resulting in sequencing data. If the step fails, the control molecule will not go forward in the process and no sequencing data will be generated. Using 100 ng of starting material, the controls yield approximately 0.2% of clusters, although this can vary based on library yield.

Table 4 In-Line Control Functions

Reagent	Function	Control	Structure of Control DNA Ends
Second Strand Marking Master Mix	End repair: Generate blunt ended fragments by 3'→5' exonuclease and 5'→3' polymerase activities	End Repair Control 1*	5' overhang at one end, 3' overhang at other end
Second Strand Marking Master Mix	End repair: Add 5'-phosphate groups needed for downstream ligation	End Repair Control 2*	Blunt with 5'-OH group
A-Tailing Mix	A-tailing: Make fragments compatible with adapters and prevent self-ligation by adding a 3'-A overhang	A-Tailing Control	Blunt with 5'-phosphate group
Ligation Mix	Ligation: Join 3'-T overhang adapters to 3'-A overhang inserts	Ligation Control	Single-base 3' 'A' base overhang

*End Repair Control 1 and End Repair Control 2 are separate controls included in the End Repair Control reagent

The control reagents can be used for various library insert sizes. Each is provided in ladders ranging from approximately 150–850 bp in 100 bp increments. Each control molecule has a unique DNA sequence, indicating both its function and size. The RTA software (v1.9, and later) recognizes these sequences and isolates the control sequences from the main body of sequencing reads. RTA reports the control sequences counts per lane in the controls tab of the RTA status.html page. For more information regarding the control read-out in the SAV, see the *Sequence Analysis Viewer User Guide* (part # 15020619).

Additional Resources

The following resources are available for TruSeq Stranded Total RNA Sample Preparation protocol guidance and sample tracking. Access these and other resources on the Illumina website at support.illumina.com/sequencing/kits.ilmn. Then, select **TruSeq Stranded Total RNA LT Sample Prep Kit Support** or **TruSeq Stranded Total RNA HT Sample Prep Kit Support**.

Resource	Description
Training	<p>Illustrates elements of the TruSeq Stranded Total RNA Sample Preparation process. Viewing these videos is recommended for new and less experienced users before starting sample preparation.</p> <ul style="list-style-type: none"> • Click Training on TruSeq Stranded Total RNA LT Sample Prep Kit Support or • Click Training on TruSeq Stranded Total RNA HT Sample Prep Kit Support
Best Practices	<p>Provides best practices specific to this protocol. Review these best practices before starting sample preparation. Topics include:</p> <ul style="list-style-type: none"> • Handling Liquids • Handling Master Mix Reagents • Handling Magnetic Beads • Avoiding Cross-Contamination • Potential DNA Contaminants • Temperature Considerations • Equipment <ul style="list-style-type: none"> • Click Best Practices on TruSeq Stranded Total RNA LT Sample Prep Kit Support or • Click Best Practices on TruSeq Stranded Total RNA HT Sample Prep Kit Support

Resource	Description
TruSeq Stranded Total RNA Sample Preparation Low Sample Experienced User Card and Lab Tracking Form (part # 15031060)	<p>Provides LS protocol instructions, but with less detail than what is provided in this user guide. New or less experienced users are advised to follow this user guide and not the EUC and Lab Tracking Form.</p> <ul style="list-style-type: none"> • Click Documentation & Literature on TruSeq Stranded Total RNA LT Sample Prep Kit Support or • Click Documentation & Literature on TruSeq Stranded Total RNA HT Sample Prep Kit Support
TruSeq Stranded Total RNA Sample Preparation High Sample Experienced User Card and Lab Tracking Form (part # 15031059)	<p>Provides HS protocol instructions, but with less detail than what is provided in this user guide. New or less experienced users are advised to follow this user guide and not the EUC and Lab Tracking Form.</p> <ul style="list-style-type: none"> • Click Documentation & Literature on TruSeq Stranded Total RNA LT Sample Prep Kit Support or • Click Documentation & Literature on TruSeq Stranded Total RNA HT Sample Prep Kit Support
TruSeq Sample Preparation Pooling Guide (part # 15042173)	<p>Provides TruSeq pooling guidelines for sample preparation. Review this guide before beginning library preparation.</p> <ul style="list-style-type: none"> • Click Documentation & Literature on TruSeq Stranded Total RNA LT Sample Prep Kit Support or • Click Documentation & Literature on TruSeq Stranded Total RNA HT Sample Prep Kit Support
Illumina Experiment Manager (IEM)	<p>Enables you to create and edit appropriate sample sheets for Illumina sequencers and analysis software and record parameters for your sample plate.</p> <p>To download the software:</p> <ul style="list-style-type: none"> • Click Downloads on TruSeq Stranded Total RNA LT Sample Prep Kit Support or • Click Downloads on TruSeq Stranded Total RNA HT Sample Prep Kit Support <p>To download the documentation:</p> <ul style="list-style-type: none"> • Click Documentation & Literature on TruSeq Stranded Total RNA LT Sample Prep Kit Support or • Click Documentation & Literature on TruSeq Stranded Total RNA HT Sample Prep Kit Support

Low Sample (LS) Protocol

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Introduction

This chapter describes the TruSeq Stranded Total RNA Sample Preparation LS protocol. Illumina recommends the following kit, sample number, and protocol combinations:

Table 5 Kit and Sample Number Recommendations

Number of Samples Processed At One Time	Recommended Kit
<24	LT
24–48	LT or HT
>48	HT

Table 6 Kit and Protocol Recommendations

Kit	Number of Samples Supported per Kit	Number of Samples Processed At One Time	Protocol
LT	48	≤48	LS
		>48	HS
HT	96	≤24	LS
		>24	HS

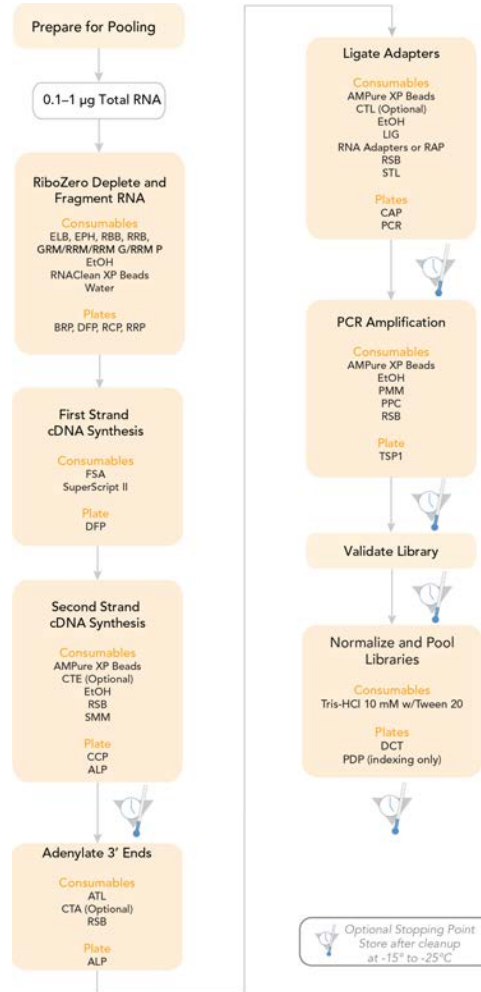
- ▶ Follow the protocol in the order described, using the specified volumes and incubation parameters.
- ▶ Before proceeding review the following:
 - Best Practices—See *Additional Resources* on page 10 for information on how to access TruSeq Stranded Total RNA Sample Preparation Best Practices on the Illumina website.
 - *TruSeq Sample Preparation Pooling Guide (part # 15042173)*—See *Additional Resources* on page 10 for information on how to download the guide from the Illumina website.

- Appendix A Supporting Information—Confirm your kit contents and make sure that you have obtained all of the requisite equipment and consumables for the LS protocol.

Sample Prep Workflow

The following illustrates the processes of the TruSeq Stranded Total RNA Sample Preparation LS protocol to prepare templates using 24 indexed adapter tubes or a RAP.

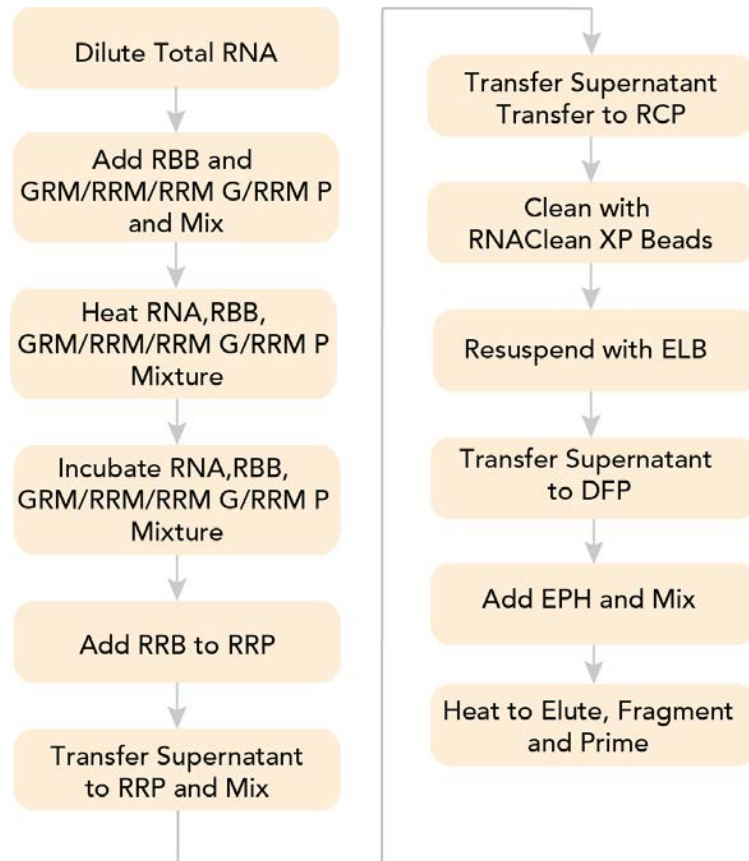
Figure 2 TruSeq Stranded Total RNA Sample Preparation LS Workflow



Ribo-Zero Deplete and Fragment RNA

This process depletes rRNA from total RNA. After the rRNA is depleted, the remaining RNA is purified, fragmented, and primed for cDNA synthesis. It is important to follow this purification procedure exactly to be sure of reproducibility. Reference the following diagram while performing the procedures:

Figure 3 TruSeq Stranded Total RNA Sample Preparation Purification Workflow



NOTE

Illumina recommends that you use 0.1–1 µg of total RNA and use PCR plates with a magnetic plate stand for this process.



NOTE

Allow the rRNA Removal Beads and the RNAClean XP Beads to fully pellet against the magnetic stand for 1 minute and 5 minutes, respectively. Remove the supernatant from the beads immediately while the beads are still pelleted against the magnetic stand. Do not allow the rRNA Removal Bead pellets to dry.



NOTE

The RNAClean XP bead wash steps use 70% Ethanol, while 80% Ethanol is used for AMPure XP bead washes.

Consumables

Item	Quantity	Storage	Supplied By
Elute, Prime, Fragment High Mix (EPH)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Elution Buffer (ELB)	1 tube per 48 reactions	2°C to 8°C	Illumina
One of the following, depending on the kit you are using: <ul style="list-style-type: none"> • Globin Removal Mix (GRM) (Ribo-Zero Globin kit contents) • rRNA Removal Mix (RRM) (Ribo-Zero Human/Mouse/Rat kit contents) • rRNA Removal Mix - Gold (RRM G) (Ribo-Zero Gold kit contents) • rRNA Removal Mix - Plant (RRM P) (Ribo-Zero Plant kit contents) 	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	-15°C to -25°C	Illumina
rRNA Binding Buffer (RBB)	1 tube per 48 reactions	-15°C to -25°C	Illumina

Item	Quantity	Storage	Supplied By
rRNA Removal Beads (RRB)	1 tube per 48 reactions	2°C to 8°C	Illumina
Barcode labels for: <ul style="list-style-type: none"> • BRP (Bind rRNA Plate) • DFP (Depleted RNA Fragmentation Plate) • RCP (RNA Clean Up Plate) • RRP (rRNA Removal Plate) 	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR Plates	4	15°C to 30°C	User
Freshly Prepared 70% Ethanol (EtOH)	200 µl per sample	15°C to 30°C	User
Microseal 'B' Adhesive Seals	2	15°C to 30°C	User
RNAClean XP Beads	99 µl per sample	2°C to 8°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	6	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	6	15°C to 30°C	User
Ultra Pure Water	Enough to dilute each total RNA sample to a final volume of 10 µl	15°C to 30°C	User

Preparation

- ▶ Remove the following from -15°C to -25°C storage and thaw them at room temperature:
 - Elute, Prime, Fragment High Mix

- One of the following, depending on the kit you are using:
 - Globin Removal Mix
 - rRNA Removal Mix
 - rRNA Removal Mix - Gold
 - rRNA Removal Mix - Plant
- rRNA Binding Buffer
- Resuspension Buffer



NOTE

The Resuspension Buffer can be stored at 2°C to 8°C after the initial thaw.

- ▶ Remove the following from 2°C to 8°C storage and let stand to bring to room temperature:
 - Elution Buffer
 - rRNA Removal Beads
- ▶ Remove the RNAClean XP beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- ▶ Pre-program the thermal cycler with the following programs:
 - Choose the pre-heat lid option and set to 100°C
 - 68°C for 5 minutes – save as **RNA Denaturation**
 - 94°C for 8 minutes, 4°C hold – save as **Elution 2 - Frag - Prime**



NOTE

For inserts larger than 120–200 bp with a median size of 150 bp or if starting with degraded total RNA, see Appendix A Alternate Fragmentation Protocols for the appropriate **Elution 2 - Frag - Prime** program settings.

- ▶ Set the centrifuge to 15°C to 25°C, if refrigerated.
- ▶ Apply a BRP barcode label to a new 96-well 0.3 ml PCR plate.
- ▶ Apply a DFP barcode label to a new 96-well 0.3 ml PCR plate.
- ▶ Apply an RCP barcode label to a new 96-well 0.3 ml PCR plate.
- ▶ Apply an RRP barcode label to a new 96-well 0.3 ml PCR plate.

Make BRP

- 1 Dilute the total RNA with nuclease-free ultra pure water to a final volume of 10 µl in the new 96-well 0.3 ml PCR plate labeled with the BRP barcode.
- 2 Add 5 µl of rRNA Binding Buffer to each well of the BRP plate.

- 3 Add 5 μ l of one of the following reagents to each well of the BRP plate, depending on the kit you are using:
 - Globin Removal Mix
 - rRNA Removal Mix
 - rRNA Removal Mix - Gold
 - rRNA Removal Mix - Plant
- 4 Gently pipette the entire volume of each well of the BRP plate up and down 6 times to mix thoroughly.
- 5 Seal the BRP plate with a Microseal 'B' adhesive seal.
- 6 Return the following to -15°C to -25°C storage:
 - rRNA Binding Buffer
 - One of the following, depending on the kit you are using:
 - Globin Removal Mix
 - rRNA Removal Mix
 - rRNA Removal Mix - Gold
 - rRNA Removal Mix - Plant

Incubate 1 BRP

- 1 Place the sealed BRP plate on the pre-programmed thermal cycler. Close the lid, then select and run the **RNA Denaturation** program.
 - a Choose the pre-heat lid option and set to 100°C
 - b 68°C for 5 minutes
- 2 After the 5 minute incubation, place the BRP plate on the bench and incubate at room temperature for 1 minute.

Make RRP

- 1 Vortex the room temperature rRNA Removal Bead tube vigorously to resuspend the beads.
- 2 Add 35 μ l of rRNA Removal Beads to each well of the new 96-well 0.3 ml PCR plate labeled with the RRP barcode.

**NOTE**

It is important not to skip this step by adding beads to the sample in the BRP plate. Adding the sample from the BRP plate to beads in the RRP plate in step 3 will ensure optimal performance.

- 3 Remove the adhesive seal from the BRP plate.
- 4 Transfer the entire contents (20 μ l) from each well of the BRP plate to the corresponding well of the RRP plate containing rRNA Removal Beads.
- 5 Adjust the pipette to 45 μ l, then with the tip of the pipette at the bottom of the well, pipette quickly up and down 20 times to mix thoroughly.

**NOTE**

It is important to pipette up and down quickly to ensure thorough mixing. Insufficient mixing leads to lower levels of rRNA depletion.

Pipetting with the tips at the bottom of the well and not pipetting the entire volume of the solution help prevent the solution from foaming. Excessive foaming leads to sample loss, because the foam is not transferred out of the plate efficiently.

- 6 Incubate the RRP plate at room temperature for 1 minute.
- 7 Place the RRP plate on the magnetic stand at room temperature for 1 minute.
- 8 Transfer all of the supernatant from each well of the RRP plate to the corresponding well of the new 96-well 0.3 ml PCR plate labeled with the RCP barcode.
- 9 Place the RCP plate on the magnetic stand at room temperature for 1 minute.

**NOTE**

If any beads remain in the wells of the RCP plate, place the RCP plate on the magnet stand for 1 minute and then transfer the supernatant to a new 0.3 ml PCR plate. Repeat as necessary until there are no beads remaining. The last 0.3 ml PCR plate will be the RCP plate used during Clean Up RCP.

- 10 Return the rRNA Removal Beads to 2°C to 8°C storage.

Clean Up RCP

- 1 Vortex the RNAClean XP beads until they are well dispersed, then add 99 μ l of well-mixed RNAClean XP beads to each well of the RCP plate containing ribosomal depleted RNA. Gently pipette the entire volume up and down 10 times to mix thoroughly.



NOTE

If starting with degraded total RNA, add 193 μ l of well-mixed RNAClean XP beads to each well of the RCP plate containing ribosomal depleted RNA.

- 2 Incubate the RCP plate at room temperature for 15 minutes.
- 3 Place the RCP plate on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells.
- 4 Remove and discard all of the supernatant from each well of the RCP plate.



NOTE

Leave the RCP plate on the magnetic stand while performing the following 70% EtOH wash steps (5–6).

- 5 With the RCP plate on the magnetic stand, add 200 μ l freshly prepared 70% EtOH to each well without disturbing the beads.
- 6 Incubate the RCP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.
- 7 Let the RCP plate stand at room temperature for 15 minutes to dry, and then remove the plate from the magnetic stand.
- 8 Centrifuge the thawed, room temperature Elution Buffer to 600 \times g for 5 seconds.
- 9 Add 11 μ l Elution Buffer to each well of the RCP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 10 Incubate the RCP plate at room temperature for 2 minutes.
- 11 Place the RCP plate on the magnetic stand at room temperature for 5 minutes.
- 12 Return the Elution Buffer to 2°C to 8°C storage.
- 13 Transfer 8.5 μ l supernatant from the RCP plate to the new 96-well 0.3 ml PCR plate labeled with the DFP barcode.

- 14 Add 8.5 μ l Elute, Prime, Fragment High Mix to each well of the DFP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 15 Seal the DFP plate with a Microseal 'B' adhesive seal.
- 16 Return the Elute, Prime, Fragment High Mix to -15°C to -25°C storage and the RNAClean XP Beads tube to 2°C to 8°C storage.

Incubate 1 DFP

- 1 Place the sealed DFP plate on the pre-programmed thermal cycler. Close the lid, then select and run the **Elution 2 - Frag - Prime** program.



NOTE

For inserts larger than 120–200 bp with a median size of 150 bp or if starting with degraded total RNA, make sure the appropriate **Elution 2 - Frag - Prime** program settings have been set. See Appendix A Alternate Fragmentation Protocols for more information.

- a Choose the pre-heat lid option and set to 100°C
 - b 94°C for 8 minutes
 - c Hold at 4°C
- 2 Remove the DFP plate from the thermal cycler when it reaches 4°C and centrifuge briefly.
 - 3 Proceed immediately to *Synthesize First Strand cDNA* on page 25.

Synthesize First Strand cDNA

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

Consumables

Item	Quantity	Storage	Supplied By
First Strand Synthesis Act D Mix (FSA)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Microseal 'B' Adhesive Seal	1	15°C to 30°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	1	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	1	15°C to 30°C	User
SuperScript II Reverse Transcriptase	1 tube	-15°C to -25°C	User



WARNING

First Strand Synthesis Act D Mix contains Actinomycin D, a toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region. Refer to the product material safety data sheet (MSDS) for detailed environmental, health, and safety information. MSDSs are available for this kit on the Illumina website at www.illumina.com/msds.

Preparation

- ▶ Remove one tube of First Strand Synthesis Act D Mix from -15°C to -25°C storage and thaw it at room temperature.

- ▶ Pre-program the thermal cycler with the following program and save as **Synthesize 1st Strand**:
 - Choose the pre-heat lid option and set to 100°C
 - 25°C for 10 minutes
 - 42°C for 15 minutes
 - 70°C for 15 minutes
 - Hold at 4°C



NOTE

The First Strand Synthesis Mix Act D with SuperScript II added is stable to additional freeze-thaw cycles and can be used for subsequent experiments. If more than six freeze-thaw cycles are anticipated, divide the First Strand Synthesis Mix Act D and SuperScript II mix into smaller aliquots and store at -15°C to -25°C.

Add FSA

- 1 Remove the adhesive seal from the DFP plate.
- 2 Centrifuge the thawed First Strand Synthesis Mix Act D tube to 600 × g for 5 seconds.
- 3 Add 50 µl SuperScript II to the First Strand Synthesis Act D Mix tube. If you are not using the entire contents of the First Strand Synthesis Act D Mix tube, add SuperScript II at a ratio of 1 µl SuperScript II for each 9 µl First Strand Synthesis Act D Mix. Mix gently, but thoroughly, and centrifuge briefly.
Label the First Strand Synthesis Mix Act D tube to indicate that the SuperScript II has been added.
- 4 Add 8 µl of First Strand Synthesis Mix Act D and SuperScript II mix to each well of the DFP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.
- 5 Seal the DFP plate with a Microseal 'B' adhesive seal and centrifuge briefly.
- 6 Return the First Strand Synthesis Mix Act D tube to -15°C to -25°C storage immediately after use.

Incubate 2 DFP

- 1 Place the sealed DFP plate on the pre-programmed thermal cycler. Close the lid, then select and run the **Synthesize 1st Strand** program.
 - a Choose the pre-heat lid option and set to 100°C
 - b 25°C for 10 minutes

- c 42°C for 15 minutes
 - d 70°C for 15 minutes
 - e Hold at 4°C
- 2 When the thermal cyclers reaches 4°C, remove the DFP plate from the thermal cyclers and proceed immediately to *Synthesize Second Strand cDNA* on page 28.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand, incorporating dUTP in place of dTTP to generate ds cDNA. The incorporation of dUTP quenches the second strand during amplification, because the polymerase does not incorporate past this nucleotide. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix. At the end of this process, you have blunt-ended cDNA.

Consumables

Item	Quantity	Storage	Supplied By
(Optional) End Repair Control (CTE)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Second Strand Marking Master Mix (SMM)	1 tube per 48 reactions	-15°C to -25°C	Illumina
ALP (Adapter Ligation Plate) Barcode Label	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR Plate	1	15°C to 30°C	User
AMPure XP Beads	90 µl per sample	2°C to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' Adhesive Seals	2	15°C to 30°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- ▶ Remove the following from -15°C to -25°C storage and thaw them at room temperature:
 - End Repair Control



NOTE

The use of the End Repair Control is optional and it can be replaced with the same volume of Resuspension Buffer.

- Second Strand Marking Master Mix
- ▶ Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- ▶ Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- ▶ Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 10 for information on how to access TruSeq Stranded Total RNA Sample Preparation Best Practices on the Illumina website.
- ▶ Pre-heat the thermal cycler to 16°C.
- ▶ Choose the thermal cycler pre-heat lid option and set to 30°C
- ▶ Apply an ALP barcode label to a new 96-well 0.3 ml PCR plate.

Add SMM

- 1 Remove the adhesive seal from the DFP plate.
- 2 Do one of the following:
 - If using the in-line control reagent:
 - Centrifuge the thawed End Repair Control tube to 600 × g for 5 seconds.
 - Dilute the End Repair Control to 1/50 in Resuspension Buffer (For example, 2 µl End Repair Control + 98 µl Resuspension Buffer) before use. Discard the diluted End Repair Control after use.
 - Add 5 µl of diluted End Repair Control to each well of the DFP plate.
 - If not using the in-line control reagent, add 5 µl of Resuspension Buffer to each well of the DFP plate.
- 3 Centrifuge the thawed Second Strand Marking Master Mix to 600 × g for 5 seconds.
- 4 Add 20 µl of thawed Second Strand Marking Master Mix to each well of the DFP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.

- 5 Seal the DFP plate with a Microseal 'B' adhesive seal.
- 6 Return the Second Strand Marking Master Mix tube to -15°C to -25°C storage after use.

Incubate 3 DFP

- 1 Place the sealed DFP plate on the pre-heated thermal cycler. Close the lid and incubate at 16°C for 1 hour.
- 2 Remove the DFP plate from the thermal cycler and place it on the bench.
- 3 Remove the adhesive seal from the DFP plate.
- 4 Let the DFP plate stand to bring it to room temperature.

Clean Up DFP

- 1 Vortex the AMPure XP beads until they are well dispersed.
- 2 Add 90 µl of well-mixed AMPure XP beads to each well of the DFP plate containing 50 µl of ds cDNA. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 3 Incubate the DFP plate at room temperature for 15 minutes.
- 4 Place the DFP plate on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells.
- 5 Remove and discard 135 µl supernatant from each well of the DFP plate.



NOTE

Leave the DFP plate on the magnetic stand while performing the following 80% EtOH wash steps (6–8).

- 6 With the DFP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.
- 7 Incubate the DFP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.
- 8 Repeat steps 6 and 7 one time for a total of two 80% EtOH washes.
- 9 Let the DFP plate stand at room temperature for 15 minutes to dry, and then remove the plate from the magnetic stand.

- 10 Centrifuge the thawed, room temperature Resuspension Buffer to $600 \times g$ for 5 seconds.
- 11 Add 17.5 μ l Resuspension Buffer to each well of the DFP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 12 Incubate the DFP plate at room temperature for 2 minutes.
- 13 Place the DFP plate on the magnetic stand at room temperature for 5 minutes.
- 14 Transfer 15 μ l supernatant (ds cDNA) from the DFP plate to the new 96-well 0.3 ml PCR plate labeled with the ALP barcode.

**SAFESTOPPING POINT**

If you do not plan to proceed immediately to *Adenylate 3' Ends* on page 32, you can safely stop the protocol here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to 7 days.

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single '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

Item	Quantity	Storage	Supplied By
(Optional) A-Tailing Control (CTA)	1 tube per 48 reactions	-15°C to -25°C	Illumina
A-Tailing Mix (ATL)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Microseal 'B' Adhesive Seal	1	15°C to 30°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	3	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	3	15°C to 30°C	User

Preparation

- ▶ Remove the following from -15°C to -25°C storage and thaw them at room temperature:

- A-Tailing Control



NOTE

The use of the A-Tailing Control is optional and it can be replaced with the same volume of Resuspension Buffer.

- A-Tailing Mix

- ▶ Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- ▶ Remove the ALP plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up DFP* on page 30.
 - Let it thaw at room temperature.
 - Centrifuge the thawed ALP plate to 280 × g for 1 minute.
 - Remove the adhesive seal from the ALP plate.
- ▶ Pre-program the thermal cycler with the following program and save as **ATAIL70**:
 - Choose the pre-heat lid option and set to 100°C
 - 37°C for 30 minutes
 - 70°C for 5 minutes
 - Hold at 4°C

Add ATL

- 1 Do one of the following:
 - If using the in-line control reagent:
 - Centrifuge the thawed A-Tailing Control tube to 600 × g for 5 seconds.
 - Dilute the A-Tailing Control to 1/100 in Resuspension Buffer (For example, 1 μl A-Tailing Control + 99 μl Resuspension Buffer) before use. Discard the diluted A-Tailing Control after use.
 - Add 2.5 μl of diluted A-Tailing Control to each well of the ALP plate.
 - If not using the in-line control reagent, add 2.5 μl of Resuspension Buffer to each well of the ALP plate.
- 2 Add 12.5 μl of thawed A-Tailing Mix to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 3 Seal the ALP plate with a Microseal 'B' adhesive seal.

Incubate 1 ALP

- 1 Place the sealed ALP plate on the pre-programmed thermal cycler. Close the lid, then select and run the **ATAIL70** program.
 - a Choose the pre-heat lid option and set to 100°C
 - b 37°C for 30 minutes
 - c 70°C for 5 minutes
 - d Hold at 4°C
- 2 When the thermal cycler temperature is 4°C, remove the ALP plate from the thermal cycler, then proceed immediately to *Ligate Adapters* on page 35.

Ligate Adapters

This process ligates multiple indexing adapters to the ends of the ds cDNA, preparing them for hybridization onto a flow cell.

Consumables

Item	Quantity	Storage	Supplied By
(Optional) Ligation Control (CTL)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Choose from the following depending on the kit you are using: <ul style="list-style-type: none"> • TruSeq Stranded Total RNA LT Sample Prep Kit contents: <ul style="list-style-type: none"> • RNA Adapter Indices (AR001–AR016, AR018–AR023, AR025, AR027) • TruSeq Stranded Total RNA HT Sample Prep Kit contents: <ul style="list-style-type: none"> • RAP (RNA Adapter Plate) 	1 tube of each index being used, per column of 8 reactions or 1 RAP	-15°C to -25°C	Illumina
Ligation Mix (LIG)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Stop Ligation Buffer (STL)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Barcode labels for: <ul style="list-style-type: none"> • CAP (Clean Up ALP Plate) • PCR (Polymerase Chain Reaction Plate) • RAP (RNA Adapter Plate) (if using the HT kit) 	1 label per plate	15°C to 30°C	Illumina

Item	Quantity	Storage	Supplied By
96-well 0.3 ml PCR Plates	2	15°C to 30°C	User
AMPure XP Beads	92 µl per sample	2°C to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	800 µl per sample	15°C to 30°C	User
Microseal 'B' Adhesive Seals	2	15°C to 30°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	4–28	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	4–28	15°C to 30°C	User

Preparation

- ▶ Remove the following from -15°C to -25°C storage and thaw them at room temperature:
 - Appropriate RNA Adapter tubes (depending on the RNA Adapter Indices being used) or the RAP.



NOTE

- Review the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 10 for information on how to download the guide from the Illumina website.
- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that are going to be combined into a common pool in the same row. Also, include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
- When indexing libraries with the RAP, arrange samples that will be pooled together in the same orientation as the indices in the RAP.



NOTE

When indexing libraries with the RAP:

- Review *Handling Adapter Plate* in the *TruSeq Sample Preparation Pooling Guide* (part # 15042173). See *Additional Resources* on page 10 for information on how to download the guide from the Illumina website.
- Illumina recommends that the RAP does not undergo more than four freeze-thaw cycles. To maximize the use of the RAP, process more than 24 samples at a time. These samples can then be pooled in any supported configuration.

- Stop Ligation Buffer



NOTE

Do not remove the Ligation Mix tube from -15°C to -25°C storage until instructed to do so in the procedures.

- Ligation Control



NOTE

The use of the Ligation Control is optional and it can be replaced with the same volume of Resuspension Buffer.

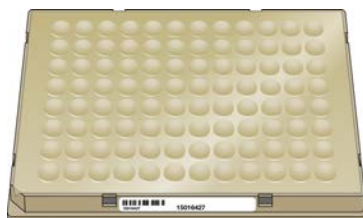
- ▶ Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- ▶ Remove the AMPure XP Beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- ▶ Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 10 for information on how to access TruSeq Stranded Total RNA Sample Preparation Best Practices on the Illumina website.
- ▶ Pre-heat the thermal cycler to 30°C.
- ▶ Choose the thermal cycler pre-heat lid option and set to 100°C
- ▶ Apply a CAP barcode label to a new 96-well 0.3 ml PCR plate.
- ▶ Apply a PCR barcode label to a new 96-well 0.3 ml PCR plate.

Add LIG

- 1 Do one of the following:
 - If using RNA Adapter tubes, centrifuge the thawed tubes to $600 \times g$ for 5 seconds.
 - If using a RAP:
 - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to make sure that they all are thawed.
 - Remove the adapter plate tape seal.

- Centrifuge the plate to $280 \times g$ for 1 minute to collect all of the adapter to the bottom of the well.
 - Remove the plastic cover. Save the cover if you are not processing the entire plate at one time.
 - If it is the first time using this RAP, apply the RAP barcode label to the plate.
- 2 Centrifuge the Ligation Control (if using Ligation Control) and Stop Ligation Buffer tubes to $600 \times g$ for 5 seconds.
 - 3 Immediately before use, remove the Ligation Mix tube from -15°C to -25°C storage.
 - 4 Remove the adhesive seal from the ALP plate.
 - 5 Do one of the following:
 - If using the in-line control reagent:
 - Dilute the Ligation Control to 1/100 in Resuspension Buffer (For example, $1 \mu\text{l}$ Ligation Control + $99 \mu\text{l}$ Resuspension Buffer) before use. Discard the diluted Ligation Control after use.
 - Add $2.5 \mu\text{l}$ of diluted Ligation Control to each well of the ALP plate.
 - If not using the in-line control reagent, add $2.5 \mu\text{l}$ of Resuspension Buffer to each well of the ALP plate.
 - 6 Add $2.5 \mu\text{l}$ of Ligation Mix to each well of the ALP plate.
 - 7 Return the Ligation Mix tube to -15°C to -25°C storage immediately after use.
 - 8 Do one of the following:
 - If using RNA Adapter tubes, add $2.5 \mu\text{l}$ of the thawed RNA Adapter Index to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
 - If using a RAP:
 - Place the RAP on the benchtop so that the part number barcode, on the long side of the plate, is facing you and the clipped corner is on the lower left.

Figure 4 Correct RAP Orientation



- Do one of the following to pierce the foil seal:
 - If using the entire plate at one time, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously. Gently, but firmly, press the clean plate over the foil seal.
 - If using only part of the plate, use the bottom of a clean eight-tube strip, with caps attached, to pierce holes in the seals of the wells that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each row or column of adapters that will be used for ligation.
 - Using an eight-tip multichannel pipette, transfer 2.5 μ l of the thawed RNA Adapter from the RAP well to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 9 Seal the ALP plate with a Microseal 'B' adhesive seal.
 - 10 Centrifuge the ALP plate to $280 \times g$ for 1 minute.

Incubate 2 ALP

- 1 Place the sealed ALP plate on the pre-heated thermal cycler. Close the lid and incubate at 30°C for 10 minutes.
- 2 Remove the ALP plate from the thermal cycler.

Add STL

- 1 Remove the adhesive seal from the ALP plate.
- 2 Add 5 μ l of Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation. Gently pipette the entire volume up and down 10 times to mix thoroughly.

Clean Up ALP

- 1 Vortex the AMPure XP Beads for at least 1 minute or until they are well dispersed.
- 2 Add 42 μ l of mixed AMPure XP Beads to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 3 Incubate the ALP plate at room temperature for 15 minutes.
- 4 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

- 5 Remove and discard 79.5 μ l supernatant from each well of the ALP plate. Take care not to disturb the beads.

**NOTE**

Leave the ALP plate on the magnetic stand while performing the following 80% EtOH wash steps (6–8).

- 6 With the ALP plate on the magnetic stand, add 200 μ l freshly prepared 80% EtOH to each well without disturbing the beads.
- 7 Incubate the ALP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.
- 8 Repeat steps 6 and 7 one time for a total of two 80% EtOH washes.
- 9 With the ALP plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes.
- 10 Remove the ALP plate from the magnetic stand.
- 11 Add 52.5 μ l Resuspension Buffer to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly or until the beads are fully resuspended.
- 12 Incubate the ALP plate at room temperature for 2 minutes.
- 13 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
- 14 Transfer 50 μ l supernatant from each well of the ALP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CAP barcode. Take care not to disturb the beads.
- 15 Vortex the AMPure XP Beads until they are well dispersed.
- 16 Add 50 μ l of mixed AMPure XP Beads to each well of the CAP plate for a second cleanup. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 17 Incubate the CAP plate at room temperature for 15 minutes.
- 18 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
- 19 Remove and discard 95 μ l supernatant from each well of the CAP plate. Take care not to disturb the beads.



NOTE

Leave the CAP plate on the magnetic stand while performing the following 80% EtOH wash steps (20–22)

- 20 With the CAP plate on the magnetic stand, add 200 μ l freshly prepared 80% EtOH to each well. Take care not to disturb the beads.
- 21 Incubate the CAP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.
- 22 Repeat steps 20 and 21 one time for a total of two 80% EtOH washes.
- 23 With the CAP plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes, and then remove the plate from the magnetic stand.
- 24 Add 22.5 μ l Resuspension Buffer to each well of the CAP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly or until the beads are fully resuspended.
- 25 Incubate the CAP plate at room temperature for 2 minutes.
- 26 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
- 27 Transfer 20 μ l supernatant from each well of the CAP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the PCR barcode. Take care not to disturb the beads.



SAFESTOPPING POINT

If you do not plan to proceed immediately to *Enrich DNA Fragments* on page 42, you can safely stop the protocol here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to seven days.

Enrich DNA Fragments

This process uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the amount of DNA in the library. The PCR is performed with a PCR Primer Cocktail that anneals to the ends of the adapters. Minimize the number of PCR cycles to avoid skewing the representation of the library.



NOTE

PCR enriches for fragments that have adapters ligated on both ends. Fragments with only one or no adapters on their ends are by-products of inefficiencies in the ligation reaction. Neither species can be used to make clusters. Fragments without any adapters cannot hybridize to surface-bound primers in the flow cell. Fragments with an adapter on only one end can hybridize to surface bound primers, but cannot form clusters.

Consumables

Item	Quantity	Storage	Supplied By
PCR Master Mix (PMM)	1 tube per 48 reactions	-15°C to -25°C	Illumina
PCR Primer Cocktail (PPC)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
TSP1 (Target Sample Plate) Barcode Label	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR Plate	1	15°C to 30°C	User
AMPure XP Beads	50 µl per sample	2°C to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' Adhesive Seals	2	15°C to 30°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	5	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- ▶ Remove the PCR Master Mix and PCR Primer Cocktail from -15°C to -25°C storage and thaw them at room temperature.
- ▶ Centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 × g for 5 seconds.
- ▶ Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- ▶ Remove the AMPure XP Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- ▶ Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 10 for information on how to access TruSeq Stranded Total RNA Sample Preparation Best Practices on the Illumina website.
- ▶ Remove the PCR plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 39.
 - Let it thaw at room temperature.
 - Centrifuge the thawed PCR plate to 280 × g for 1 minute.
 - Remove the adhesive seal from the thawed PCR plate.
- ▶ Pre-program the thermal cycler with the following program and save as **PCR**:
 - Choose the pre-heat lid option and set to 100°C
 - 98°C for 30 seconds
 - 15 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
- ▶ Apply a TSP1 barcode label to a new 96-well 0.3 ml PCR plate.

Make PCR


- 1 Add 5 μ l of thawed PCR Primer Cocktail to each well of the PCR plate.
- 2 Add 25 μ l of thawed PCR Master Mix to each well of the PCR plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 3 Seal the PCR plate with a Microseal 'B' adhesive seal.

Amp PCR

- 1 Place the sealed PCR plate on the pre-programmed thermal cycler. Close the lid, then select and run **PCR** to amplify the plate.
 - a Choose the pre-heat lid option and set to 100°C
 - b 98°C for 30 seconds
 - c 15 cycles of:
 - 98°C for 10 seconds
 - 60°C for 30 seconds
 - 72°C for 30 seconds
 - d 72°C for 5 minutes
 - e Hold at 4°C

Clean Up PCR

- 1 Remove the adhesive seal from the PCR plate.
- 2 Vortex the AMPure XP Beads until they are well dispersed.
- 3 Do one of the following, depending on the adapter type used:
 - If using the RNA Adapter tubes, add 50 μ l of the mixed AMPure XP Beads to each well of the PCR plate containing 50 μ l of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.
 - If using the RAP, add 47.5 μ l of the mixed AMPure XP Beads to each well of the PCR plate containing 50 μ l of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 4 Incubate the PCR plate at room temperature for 15 minutes.

- 5 Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
- 6 Remove and discard 95 μ l supernatant from each well of the PCR plate.
- 7  **NOTE**
Leave the PCR plate on the magnetic stand while performing the following 80% EtOH wash steps (7–9).
- 8 With the PCR plate on the magnetic stand, add 200 μ l freshly prepared 80% EtOH to each well without disturbing the beads.
- 9 Incubate the PCR plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.
- 10 Repeat steps 7 and 8 one time for a total of two 80% EtOH washes.
- 11 With the PCR plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes, and then remove the plate from the magnetic stand.
- 12 Add 32.5 μ l Resuspension Buffer to each well of the PCR plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 13 Incubate the PCR plate at room temperature for 2 minutes.
- 14 Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
- 15 Transfer 30 μ l supernatant from each well of the PCR plate to the corresponding well of the new 0.3 ml PCR plate labeled with the TSP1 barcode.

**SAFESTOPPING POINT**

If you do not plan to proceed immediately to *Validate Library* on page 46, you can safely stop the protocol here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to 7 days.

Validate Library

Illumina recommends performing the following procedures for quality control analysis on your sample library and quantification of the DNA library templates.

Quantify Libraries

To achieve the highest quality data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of the flow cell. Optimizing cluster densities requires accurate quantitation of DNA library templates. Quantify your libraries using qPCR according to the Illumina *Sequencing Library qPCR Quantification Guide* (part # 11322363).

Quality Control

- 1 Load 1 μ l of the resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA-specific chip such as the Agilent DNA 1000.
- 2 Check the size and purity of the sample. The final product should be a band at approximately 260 bp.

Figure 5 Example of TruSeq Stranded Total RNA Sample Preparation Library Size Distribution

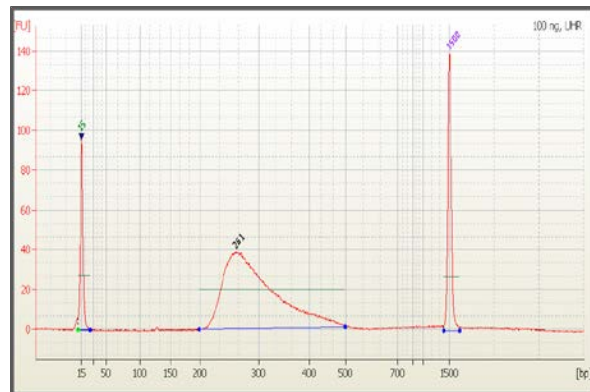
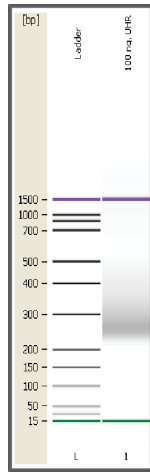


Figure 6 TruSeq Stranded Total RNA Sample Preparation 260 bp PCR Product



Normalize and Pool Libraries

This process describes how to prepare DNA templates for cluster generation. Indexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. DNA libraries not intended for pooling are normalized to 10 nM in the DCT plate.

Consumables

Item	Quantity	Storage	Supplied By
Barcode labels for: <ul style="list-style-type: none"> • DCT (Diluted Cluster Template) • PDP (Pooled DCT Plate) (for pooling only) 	1 label per plate	15°C to 30°C	Illumina
96-well MIDI plates	2 (second plate for pooling only, if pooling > 40 samples)	15°C to 30°C	User
96-well 0.3 ml PCR plate (for pooling only, if pooling ≤ 40 samples)	1	15°C to 30°C	User
Microseal 'B' Adhesive Seals	2	15°C to 30°C	User
Tris-HCl 10 mM, pH8.5 with 0.1% Tween 20	Enough to normalize the concentration of each sample library to 10 nM	15°C to 30°C	User

Preparation

- ▶ Remove the TSP1 plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 44.
 - Let it thaw at room temperature.
 - Centrifuge the thawed TSP1 plate to 280 × g for 1 minute.
 - Remove the adhesive seal from the thawed TSP1 plate.
- ▶ Apply a DCT barcode label to a new 96-well MIDI plate.
- ▶ [For pooling only] Apply a PDP barcode label to a new 96-well 0.3 ml PCR plate if pooling ≤ 40 samples or a 96-well MIDI plate if pooling > 40 samples.

Make DCT

- 1 Transfer 10 µl of sample library from each well of the TSP1 plate to the corresponding well of the new MIDI plate labeled with the DCT barcode.
- 2 Normalize the concentration of sample library in each well of the DCT plate to 10 nM using Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20.



NOTE

Depending on the yield quantification data of each sample library, the final volume in the DCT plate can vary from 10–400 µl.


- 3 Gently pipette the entire normalized sample library volume up and down 10 times to mix thoroughly.
- 4 Depending on the type of library you want to generate, do one of the following:
 - For non-pooled libraries, the protocol stops here. Do one of the following:
 - Proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina platform.
 - Seal the DCT plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C.
 - For pooled libraries, proceed to *Make PDP (for pooling only)*.

Make PDP (for pooling only)



NOTE

Do not make a PDP plate if you are not pooling samples.

- 1 Determine the number of samples to be combined together for each pool.
 -  NOTE
Note the sample that is in each well, to avoid pooling two samples with the same index.
- 2 Do one of the following:
 - If pooling 2–24 samples:
 - Transfer 10 μ l of each normalized sample library to be pooled from the DCT plate to one well of the new 0.3 ml PCR plate labeled with the PDP barcode. The total volume in each well of the PDP plate is 10 X the number of combined sample libraries and 20–240 μ l (2–24 libraries). For example, the volume for 2 samples is 20 μ l, the volume for 12 samples is 120 μ l, or the volume for 24 samples is 240 μ l.
 - If pooling 25–96 samples:
 - Using a multichannel pipette, transfer 5 μ l of each normalized sample library in column 1 of the DCT plate to column 1 of the new 0.3 ml PCR or MIDI plate labeled with the PDP barcode.
 - Transfer 5 μ l of each normalized sample library in column 2 of the DCT plate to column 1 of the PDP plate.
 - Repeat the transfer for as many times as there are remaining columns in the DCT plate. The result is a PDP plate with pooled samples in column 1. Gently pipette the entire volume of each well of column 1 up and down 10 times to mix thoroughly.
 - Combine the contents of each well of column 1 into well A2 of the PDP plate for the final pool.
- 3 Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 4 Do one of the following:
 - Proceed to cluster generation. For more information, see the user guide for your Illumina sequencer.
 - Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C.

High Sample (HS) Protocol

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Introduction

This chapter describes the TruSeq Stranded Total RNA Sample Preparation HS protocol. Illumina recommends the following kit, sample number, and protocol combinations:

Table 7 Kit and Sample Number Recommendations

Number of Samples Processed At One Time	Recommended Kit
<24	LT
24–48	LT or HT
>48	HT

Table 8 Kit and Protocol Recommendations

Kit	Number of Samples Supported per Kit	Number of Samples Processed At One Time	Protocol
LT	48	≤48	LS
		>48	HS
HT	96	≤24	LS
		>24	HS

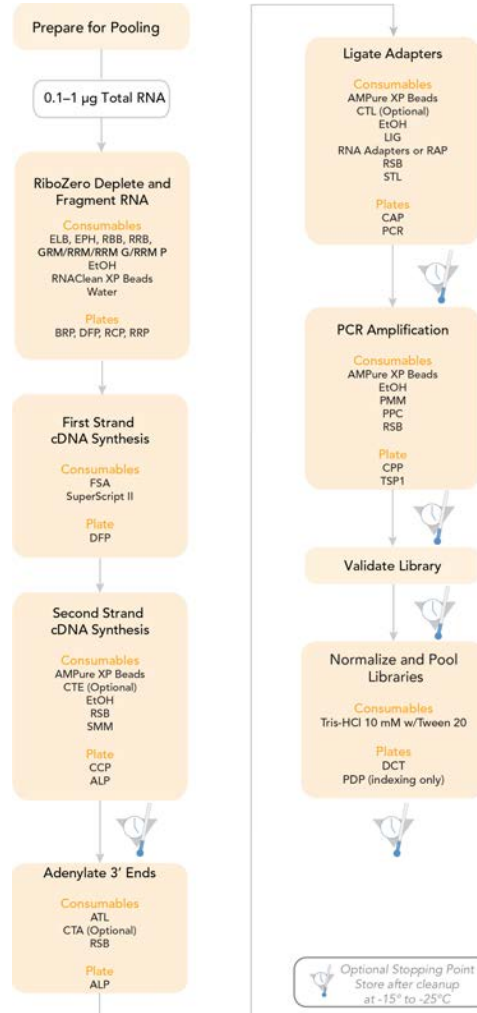
- ▶ Follow the protocol in the order described, using the specified volumes and incubation parameters.
- ▶ Before proceeding review the following:
 - Best Practices—See *Additional Resources* on page 10 for information on how to access TruSeq Stranded Total RNA Sample Preparation Best Practices on the Illumina website.
 - *TruSeq Sample Preparation Pooling Guide (part # 15042173)*—See *Additional Resources* on page 10 for information on how to download the guide from the Illumina website.

- Appendix A Supporting Information—To confirm your kit contents and make sure that you have obtained all of the requisite equipment and consumables for the HS protocol.

Sample Prep Workflow

The following illustrates the processes of the TruSeq Stranded Total RNA Sample Preparation HS protocol to prepare templates using 24 indexed adapter tubes or a RAP.

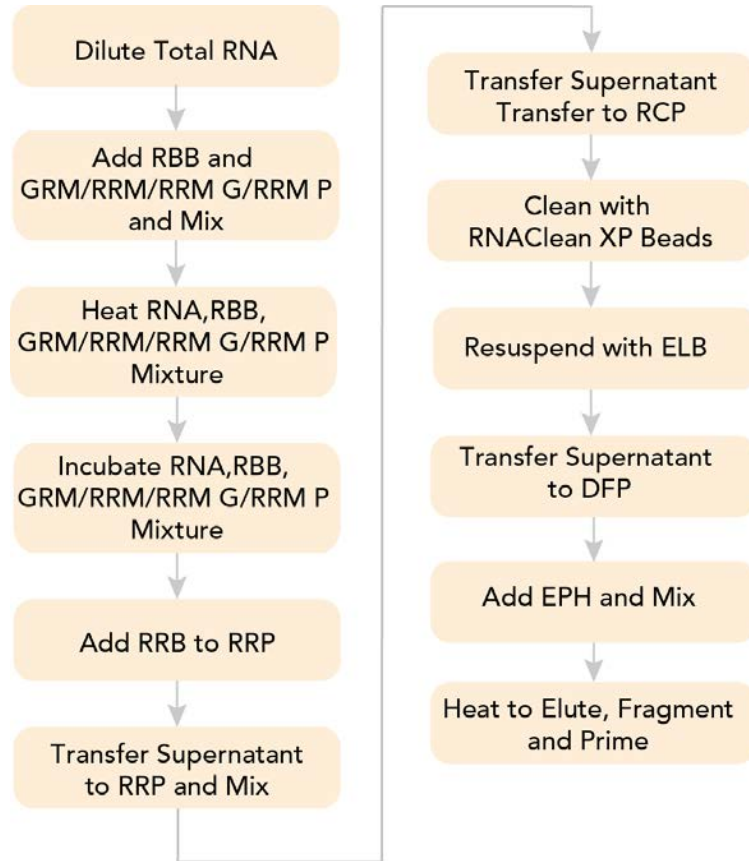
Figure 7 TruSeq Stranded Total RNA Sample Preparation HS Workflow



Ribo-Zero Deplete and Fragment RNA

This process depletes rRNA from total RNA. After the rRNA is depleted, the remaining RNA is purified, fragmented, and primed for cDNA synthesis. It is important to follow this purification procedure exactly to be sure of reproducibility. Reference the following diagram while performing the procedures:

Figure 8 TruSeq Stranded Total RNA Sample Preparation Purification Workflow



NOTE

Illumina recommends that you use 0.1–1 µg of total RNA and use PCR plates with a magnetic plate stand for this process.

**NOTE**

Allow the rRNA Removal Beads and the RNAClean XP Beads to fully pellet against the magnetic stand for 1 minute and 5 minutes, respectively. Remove the supernatant from the beads immediately while the beads are still pelleted against the magnetic stand. Do not allow the rRNA Removal Bead pellets to dry.

**NOTE**

The RNAClean XP bead wash steps use 70% Ethanol, while 80% Ethanol is used for AMPure XP bead washes.

Item	Quantity	Storage	Supplied By
Elute, Prime, Fragment High Mix (EPH)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Elution Buffer (ELB)	1 tube per 48 reactions	2°C to 8°C	Illumina
One of the following, depending on the kit you are using: <ul style="list-style-type: none"> • Globin Removal Mix (GRM) (Ribo-Zero Globin kit contents) • rRNA Removal Mix (RRM) (Ribo-Zero Human/Mouse/Rat kit contents) • rRNA Removal Mix - Gold (RRM G) (Ribo-Zero Gold kit contents) • rRNA Removal Mix - Plant (RRM P) (Ribo-Zero Plant kit contents) 	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	-15°C to -25°C	Illumina
rRNA Binding Buffer (RBB)	1 tube per 48 reactions	-15°C to -25°C	Illumina
rRNA Removal Beads (RRB)	1 tube per 48 reactions	2°C to 8°C	Illumina

Item	Quantity	Storage	Supplied By
Barcode labels for: <ul style="list-style-type: none"> • BRP (Bind rRNA Plate) • DFP (Depleted RNA Fragmentation Plate) • RCP (RNA Clean Up Plate) • RRP (rRNA Removal Plate) 	1 label per plate	15°C to 30°C	Illumina
96-well HSP Plates	2	15°C to 30°C	User
96-well MIDI Plates	2	15°C to 30°C	User
Freshly Prepared 70% Ethanol (EtOH)	200 µl per sample	15°C to 30°C	User
Microseal 'B' Adhesive Seals	5	15°C to 30°C	User
RNAClean XP Beads	99 µl per sample	2°C to 8°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	6	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	6	15°C to 30°C	User
Ultra Pure Water	Enough to dilute each total RNA sample to a final volume of 10 µl	15°C to 30°C	User

Preparation

- ▶ Remove the following from -15°C to -25°C storage and thaw them at room temperature:
 - Elute, Prime, Fragment High Mix
 - One of the following, depending on the kit you are using:
 - Globin Removal Mix
 - rRNA Removal Mix
 - rRNA Removal Mix - Gold
 - rRNA Removal Mix - Plant

- rRNA Binding Buffer
- Resuspension Buffer



NOTE

The Resuspension Buffer can be stored at 2°C to 8°C after the initial thaw.

- ▶ Remove the following from 2°C to 8°C storage and let stand to bring to room temperature:
 - Elution Buffer
 - rRNA Removal Beads
- ▶ Remove the RNAClean XP beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- ▶ Pre-program the thermal cycler with the following programs:
 - Choose the pre-heat lid option and set to 100°C
 - 68°C for 5 minutes – save as **RNA Denaturation**
 - 94°C for 8 minutes, 4°C hold – save as **Elution 2 - Frag - Prime**



NOTE

For inserts larger than 120–200 bp with a median size of 150 bp or if starting with degraded total RNA, see Appendix A Alternate Fragmentation Protocols for the appropriate **Elution 2 - Frag - Prime** program settings.

- ▶ Set the centrifuge to 15°C to 25°C, if refrigerated.
- ▶ Apply a BRP barcode label to a new 96-well HSP plate.
- ▶ Apply a DFP barcode label to a new 96-well HSP plate.
- ▶ Apply an RCP barcode label to a new 96-well MIDI plate.
- ▶ Apply an RRP barcode label to a new 96-well MIDI plate.

Make BRP

- 1 Dilute the total RNA with nuclease-free ultra pure water to a final volume of 10 µl in the new 96-well HSP plate labeled with the BRP barcode.
- 2 Add 5 µl of rRNA Binding Buffer to each well of the BRP plate.
- 3 Add 5 µl of one of the following reagents to each well of the BRP plate, depending on the kit you are using:
 - Globin Removal Mix
 - rRNA Removal Mix
 - rRNA Removal Mix - Gold
 - rRNA Removal Mix - Plant

- 4 Mix the contents of the BRP plate thoroughly as follows:
 - a Seal the BRP plate with a Microseal 'B' adhesive seal.
 - b Shake the BRP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.
- 5 Centrifuge the BRP plate to $280 \times g$ for 1 minute.
- 6 Return the following to -15°C to -25°C storage:
 - rRNA Binding Buffer
 - One of the following, depending on the kit you are using:
 - Globin Removal Mix
 - rRNA Removal Mix
 - rRNA Removal Mix - Gold
 - rRNA Removal Mix - Plant

Incubate 1 BRP

- 1 Place the sealed BRP plate on the pre-programmed thermal cycler. Close the lid, then select and run the **RNA Denaturation** program.
 - a Choose the pre-heat lid option and set to 100°C
 - b 68°C for 5 minutes
- 2 After the 5 minute incubation, place the BRP plate on the bench and incubate at room temperature for 1 minute.

Make RRP

- 1 Vortex the room temperature rRNA Removal Bead tube vigorously to resuspend the beads.
- 2 Add $35 \mu\text{l}$ of rRNA Removal Beads to each well of the new 96-well MIDI plate labeled with the RRP barcode.



NOTE

It is important not to skip this step by adding beads to the sample in the BRP plate. Adding the sample from the BRP plate to beads in the RRP plate in step 3 will ensure optimal performance.

- 3 Remove the adhesive seal from the BRP plate.

- 4 Transfer the entire contents (20 μ l) from each well of the BRP plate to the corresponding well of the RRP plate containing rRNA Removal Beads.
- 5 Mix the contents of the RRP plate thoroughly as follows:
 - a Seal the RRP plate with a Microseal 'B' adhesive seal.
 - b Shake the RRP plate on a microplate shaker continuously at 1000 rpm for 1 minute.
- 6 Remove the adhesive seal from the RRP plate.
- 7 Place the RRP plate on the magnetic stand at room temperature for 1 minute.
- 8 Transfer all of the supernatant from each well of the RRP plate to the corresponding well of the new 96-well MIDI plate labeled with the RCP barcode.
- 9 Place the RCP plate on the magnetic stand at room temperature for 1 minute.

**NOTE**

If any beads remain in the wells of the RCP plate, place the RCP plate on the magnet stand for 1 minute and then transfer the supernatant to a new MIDI plate. Repeat as necessary until there are no beads remaining. The last MIDI plate will be the RCP plate used during Clean Up RCP.

- 10 Return the rRNA Removal Beads to 2°C to 8°C storage.

Clean Up RCP

- 1 Vortex the RNAClean XP beads until they are well dispersed, then add 99 μ l of well-mixed RNAClean XP beads to each well of the RCP plate containing ribosomal depleted RNA. Mix thoroughly as follows:

**NOTE**

If starting with degraded total RNA, add 193 μ l of well-mixed RNAClean XP beads to each well of the RCP plate containing ribosomal depleted RNA.

- a Seal the RCP plate with a Microseal 'B' adhesive seal.
 - b Shake the RCP plate on a microplate shaker at 1800 rpm for 2 minutes.
- 2 Incubate the RCP plate at room temperature for 15 minutes.
- 3 Remove the adhesive seal from the RCP plate.
- 4 Place the RCP plate on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells.

- 5 Remove and discard all of the supernatant from each well of the RCP plate.

**NOTE**

Leave the RCP plate on the magnetic stand while performing the following 70% EtOH wash steps (6–7).

- 6 With the RCP plate on the magnetic stand, add 200 μ l freshly prepared 70% EtOH to each well without disturbing the beads.
- 7 Incubate the RCP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.
- 8 Let the RCP plate stand at room temperature for 15 minutes to dry, and then remove the RCP plate from the magnetic stand.
- 9 Centrifuge the thawed, room temperature Elution Buffer to 600 \times g for 5 seconds.
- 10 Add 11 μ l Elution Buffer to each well of the RCP plate. Mix thoroughly as follows:
 - a Seal the RCP plate with a Microseal 'B' adhesive seal.
 - b Shake the RCP plate on a microplate shaker at 1800 rpm for 2 minutes.
- 11 Incubate the RCP plate at room temperature for 2 minutes.
- 12 Centrifuge the RCP plate to 280 \times g for 1 minute.
- 13 Remove the adhesive seal from the RCP plate.
- 14 Place the RCP plate on the magnetic stand at room temperature for 5 minutes.
- 15 Return the Elution Buffer to 2°C to 8°C storage.
- 16 Transfer 8.5 μ l supernatant from the RCP plate to the new 96-well HSP plate labeled with the DFP barcode.
- 17 Add 8.5 μ l Elute, Prime, Fragment High Mix to each well of the DFP plate. Mix thoroughly as follows:
 - a Seal the DFP plate with a Microseal 'B' adhesive seal.
 - b Shake the DFP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.
- 18 Return the Elute, Prime, Fragment High Mix to -15°C to -25°C storage and the RNAClean XP Beads tube to 2°C to 8°C storage.

Incubate 1 DFP

- 1 Place the sealed DFP plate on the pre-programmed thermal cycler. Close the lid and select **Elution 2 - Frag - Prime** to fragment and prime the RNA.



NOTE

If starting with degraded total RNA, make sure the appropriate **Elution 2 - Frag - Prime** program settings have been set. See Appendix A Alternate Fragmentation Protocols for more information.

- a Choose the pre-heat lid option and set to 100°C
 - b 94°C for 8 minutes
 - c Hold at 4°C
- 2 Remove the DFP plate from the thermal cycler when it reaches 4°C and centrifuge briefly.
 - 3 Proceed immediately to *Synthesize First Strand cDNA* on page 63.

Synthesize First Strand cDNA

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

Consumables

Item	Quantity	Storage	Supplied By
First Strand Synthesis Act D Mix (FSA)	1 tube	-15°C to -25°C	Illumina
Microseal 'B' Adhesive Seal	1	15°C to 30°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	1	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	1	15°C to 30°C	User
SuperScript II Reverse Transcriptase	1 tube	-15°C to -25°C	User



WARNING

First Strand Synthesis Act D Mix contains Actinomycin D, a toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region. Refer to the product material safety data sheet (MSDS) for detailed environmental, health, and safety information. MSDSs are available for this kit on the Illumina website at www.illumina.com/msds.

Preparation

- ▶ Remove one tube of First Strand Synthesis Act D Mix from -15°C to -25°C storage and thaw it at room temperature.

- ▶ Pre-program the thermal cycler with the following program and save as **Synthesize 1st Strand**:
 - Choose the pre-heat lid option and set to 100°C
 - 25°C for 10 minutes
 - 42°C for 15 minutes
 - 70°C for 15 minutes
 - Hold at 4°C
- ▶ Make sure that the microplate shaker is properly calibrated to 1000 rpm using a stroboscope.

**NOTE**

The First Strand Synthesis Mix Act D with SuperScript II added is stable to additional freeze-thaw cycles and can be used for subsequent experiments. If more than six freeze-thaw cycles are anticipated, divide the First Strand Synthesis Mix Act D and SuperScript II mix into smaller aliquots and store at -15°C to -25°C.

Add FSA

- 1 Remove the adhesive seal from the DFP plate.
- 2 Centrifuge the thawed First Strand Synthesis Mix Act D tube to 600 × g for 5 seconds.
- 3 Add 50 µl SuperScript II to the First Strand Synthesis Act D Mix tube. Mix gently, but thoroughly and centrifuge briefly. If you are not using the entire contents of the First Strand Synthesis Act D Mix tube, add SuperScript II at a ratio of 1 µl SuperScript II for each 9 µl First Strand Synthesis Act D Mix.
Label the First Strand Synthesis Mix Act D tube to indicate that the SuperScript II has been added.
- 4 Add 8 µl of First Strand Synthesis Mix Act D and SuperScript II mix to each well of the DFP plate. Mix thoroughly as follows:
 - a Seal the DFP plate with a Microseal 'B' adhesive seal.
 - b Shake the DFP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.
- 5 Return the First Strand Synthesis Mix Act D tube to -15°C to -25°C storage immediately after use.

Incubate 2 DFP

- 1 Place the sealed DFP plate on the pre-programmed thermal cycler. Close the lid and select **Synthesize 1st Strand**.
 - a Choose the pre-heat lid option and set to 100°C
 - b 25°C for 10 minutes
 - c 42°C for 15 minutes
 - d 70°C for 15 minutes
 - e Hold at 4°C
- 2 When the thermal cycler reaches 4°C, remove the DFP plate from the thermal cycler and proceed immediately to *Synthesize Second Strand cDNA* on page 66.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand, incorporating dUTP in place of dTTP to generate ds cDNA. The incorporation of dUTP quenches the second strand during amplification, because the polymerase does not incorporate past this nucleotide. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix. At the end of this process, you have blunt-ended cDNA.

Consumables

Item	Quantity	Storage	Supplied By
(Optional) End Repair Control (CTE)	1 tube per 48 reactions	2°C to 8°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Second Strand Marking Master Mix (SMM)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Barcode labels for: <ul style="list-style-type: none"> • ALP (Adapter Ligation Plate) • CCP (cDNA Clean Up Plate) • IMP (Insert Modification Plate) 	1 label per plate	15°C to 30°C	Illumina
96-well MIDI Plates	2	15°C to 30°C	User
AMPure XP Beads	90 µl per sample	2°C to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' Adhesive Seals	4	15°C to 30°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- ▶ Remove the following from -15°C to -25°C storage and thaw them at room temperature:
 - End Repair Control



NOTE

The use of the End Repair Control is optional and it can be replaced with the same volume of Resuspension Buffer.

- Second Strand Marking Master Mix
- ▶ Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- ▶ Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- ▶ Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 10 for information on how to access TruSeq Stranded Total RNA Sample Preparation Best Practices on the Illumina website.
- ▶ Pre-heat the thermal cycler to 16°C.
- ▶ Choose the thermal cycler pre-heat lid option and set to 30°C
- ▶ Apply an ALP barcode label to a new 96-well MIDI plate.
- ▶ Apply a CCP barcode label to a new 96-well MIDI plate.

Add SMM

- 1 Remove the adhesive seal from the DFP plate.
- 2 Do one of the following:
 - If using the in-line control reagent:
 - Centrifuge the thawed End Repair Control tube to 600 × g for 5 seconds.
 - Dilute the End Repair Control to 1/50 in Resuspension Buffer (For example, 2 µl End Repair Control + 98 µl Resuspension Buffer) before use. Discard the diluted End Repair Control after use.
 - Add 5 µl of diluted End Repair Control to each well of the DFP plate.
 - If not using the in-line control reagent, add 5 µl of Resuspension Buffer to each well of the DFP plate.
- 3 Centrifuge the thawed Second Strand Marking Master Mix to 600 × g for 5 seconds.

- 4 Add 20 μ l of thawed Second Strand Marking Master Mix to each well of the DFP plate. Mix thoroughly as follows:
 - a Seal the DFP plate with a Microseal 'B' adhesive seal.
 - b Shake the DFP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.
- 5 Return the Second Strand Marking Master Mix tube to -15°C to -25°C storage after use.

Incubate 3 DFP

- 1 Place the sealed DFP plate on the pre-heated thermal cycler. Close the lid and incubate at 16°C for 1 hour.
- 2 Remove the DFP plate from the thermal cycler and place it on the bench.
- 3 Remove the adhesive seal from the DFP plate.
- 4 Let the DFP plate stand to bring it to room temperature.

Clean Up DFP

- 1 Vortex the AMPure XP beads until they are well dispersed.
- 2 Add 90 μ l of well-mixed AMPure XP beads to each well of the new MIDI plate labeled with the CCP barcode.
- 3 Transfer the entire contents from each well of the DFP plate to the corresponding well of the CCP plate containing AMPure XP beads. Mix thoroughly as follows:
 - a Seal the CCP plate with a Microseal 'B' adhesive seal.
 - b Shake the CCP plate on a microplate shaker at 1800 rpm for 2 minutes.
- 4 Incubate the CCP plate at room temperature for 15 minutes.
- 5 Centrifuge the CCP plate to 280 \times g for 1 minute.
- 6 Remove the adhesive seal from the CCP plate.
- 7 Place the CCP plate on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells.
- 8 Remove and discard 135 μ l supernatant from each well of the CCP plate.



NOTE

Leave the CCP plate on the magnetic stand while performing the following 80% EtOH wash steps (9–11).

- 9 With the CCP plate on the magnetic stand, add 200 μ l freshly prepared 80% EtOH to each well without disturbing the beads.
- 10 Incubate the CCP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.
- 11 Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.
- 12 Let the CCP plate stand at room temperature for 15 minutes to dry, and then remove the CCP plate from the magnetic stand.
- 13 Centrifuge the thawed, room temperature Resuspension Buffer to $600 \times g$ for 5 seconds.
- 14 Add 17.5 μ l Resuspension Buffer to each well of the CCP plate. Mix thoroughly as follows:
 - a Seal the CCP plate with a Microseal 'B' adhesive seal.
 - b Shake the CCP plate on a microplate shaker at 1800 rpm for 2 minutes.
- 15 Incubate the CCP plate at room temperature for 2 minutes.
- 16 Centrifuge the CCP plate to $280 \times g$ for 1 minute.
- 17 Remove the adhesive seal from the CCP plate.
- 18 Place the CCP plate on the magnetic stand at room temperature for 5 minutes.
- 19 Transfer 15 μ l supernatant (ds cDNA) from the CCP plate to the new MIDI plate labeled with the ALP barcode.



SAFESTOPPING POINT

If you do not plan to proceed immediately to *Adenylate 3' Ends* on page 70, you can safely stop the protocol here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to 7 days.

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single '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

Item	Quantity	Storage	Supplied By
(Optional) A-Tailing Control (CTA)	1 tube per 48 reactions	-15°C to -25°C	Illumina
A-Tailing Mix (ATL)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Ice	As needed to place a plate on	-15°C to -25°C	User
Microseal 'B' Adhesive Seal	1	15°C to 30°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	3	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	3	15°C to 30°C	User

Preparation

- ▶ Remove the following from -15°C to -25°C storage and thaw them at room temperature:

- A-Tailing Control



NOTE

The use of the A-Tailing Control is optional and it can be replaced with the same volume of Resuspension Buffer.

- A-Tailing Mix
- ▶ Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- ▶ Remove the ALP plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up DFP* on page 68.
 - Let it thaw at room temperature.
 - Centrifuge the thawed ALP plate to 280 × g for 1 minute.
 - Remove the adhesive seal from the ALP plate.
- ▶ Pre-heat two microheating systems: system 1 to 37°C and system 2 to 70°C.
- ▶ Prepare ice to cool the plate.

Add ATL

- 1 Do one of the following:
 - If using the in-line control reagent:
 - Centrifuge the thawed A-Tailing Control tube to 600 × g for 5 seconds.
 - Dilute the A-Tailing Control to 1/100 in Resuspension Buffer (For example, 1 μl A-Tailing Control + 99 μl Resuspension Buffer) before use. Discard the diluted A-Tailing Control after use.
 - Add 2.5 μl of diluted A-Tailing Control to each well of the ALP plate.
 - If not using the in-line control reagent, add 2.5 μl of Resuspension Buffer to each well of the ALP plate.
- 2 Add 12.5 μl of thawed A-Tailing Mix to each well of the ALP plate. Mix thoroughly as follows:
 - a Seal the ALP plate with a Microseal 'B' adhesive seal.
 - b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.
- 3 Centrifuge the ALP plate to 280 × g for 1 minute.

Incubate 1 ALP

- 1 Place the sealed ALP plate on the pre-heated microheating system 1. Close the lid and incubate at 37°C for 30 minutes.
- 2 Immediately after the 37°C incubation, remove the ALP plate from system 1 and place the plate on the pre-heated microheating system 2. Close the lid and incubate at 70°C for 5 minutes.
- 3 Set the microheating system 1 to 30°C in preparation for *Ligate Adapters*.
- 4 Immediately remove the ALP plate from the microheating system 2 and place the plate on ice for 1 minute.
- 5 Proceed immediately to *Ligate Adapters* on page 73.

Ligate Adapters

This process ligates indexing adapters to the ends of the ds cDNA, preparing them for hybridization onto a flow cell.

Consumables

Item	Quantity	Storage	Supplied By
(Optional) Ligation Control (CTL)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Choose from the following depending on the kit you are using: <ul style="list-style-type: none"> • TruSeq Stranded Total RNA LT Sample Prep Kit contents: <ul style="list-style-type: none"> • RNA Adapter Indices (AR001–AR016, AR018–AR023, AR025, AR027) • TruSeq Stranded Total RNA HT Sample Prep Kit contents: <ul style="list-style-type: none"> • RAP (RNA Adapter Plate) 	1 tube of each index being used, per column of 8 reactions or 1 RAP	-15°C to -25°C	Illumina
Ligation Mix (LIG)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Stop Ligation Buffer (STL)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Barcode labels for: <ul style="list-style-type: none"> • CAP (Clean Up ALP Plate) • PCR (Polymerase Chain Reaction Plate) • RAP (RNA Adapter Plate) (if using the HT kit) 	1 label per plate	15°C to 30°C	Illumina

Item	Quantity	Storage	Supplied By
96-well HSP Plate	1	15°C to 30°C	User
96-well MIDI Plate	1	15°C to 30°C	User
AMPure XP Beads	92 µl per sample	2°C to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	800 µl per sample	15°C to 30°C	User
Microseal 'B' Adhesive Seals	7	15°C to 30°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	4–28	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	4–28	15°C to 30°C	User

Preparation

- ▶ Remove the following from -15°C to -25°C storage and thaw them at room temperature:
 - Appropriate RNA Adapter tubes (depending on the RNA Adapter Indices being used) or the RAP.



NOTE

- Review the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 10 for information on how to download the guide from the Illumina website.
- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that are going to be combined into a common pool in the same row. Also, include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
- When indexing libraries with the RAP, arrange samples that will be pooled together in the same orientation as the indices in the RAP.

**NOTE**

When indexing libraries with the RAP:

- Review *Handling Adapter Plate* in the *TruSeq Sample Preparation Pooling Guide* (part # 15042173). See *Additional Resources* on page 10 for information on how to download the guide from the Illumina website.
- Illumina recommends that the RAP does not undergo more than four freeze-thaw cycles. To maximize the use of the RAP, process more than 24 samples at a time. These samples can then be pooled in any supported configuration.

- Stop Ligation Buffer

**NOTE**

Do not remove the Ligation Mix tube from -15°C to -25°C storage until instructed to do so in the procedures.

- Ligation Control

**NOTE**

The use of the Ligation Control is optional and it can be replaced with the same volume of Resuspension Buffer.

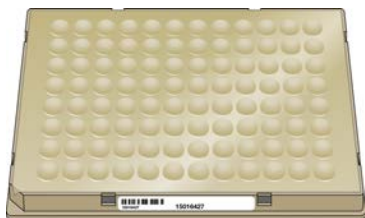
- ▶ Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- ▶ Remove the AMPure XP Beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- ▶ Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 10 for information on how to access TruSeq Stranded Total RNA Sample Preparation Best Practices on the Illumina website.
- ▶ Pre-heat the microheating system 1 to 30°C.
- ▶ Apply a CAP barcode label to a new 96-well MIDI plate.
- ▶ Apply a PCR barcode label to a new 96-well HSP plate.

Add LIG

- 1 Do one of the following:
 - If using RNA Adapter tubes, centrifuge the thawed tubes to $600 \times g$ for 5 seconds.
 - If using a RAP:
 - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to make sure that they all are thawed.
 - Remove the adapter plate tape seal.

- Centrifuge the plate to $280 \times g$ for 1 minute to collect all of the adapter to the bottom of the well.
 - Remove the plastic cover. Save the cover if you are not processing the entire plate at one time.
 - If it is the first time using this RAP, apply the RAP barcode label to the plate.
- 2 Centrifuge the Ligation Control (if using Ligation Control) and Stop Ligation Buffer tubes to $600 \times g$ for 5 seconds.
 - 3 Immediately before use, remove the Ligation Mix tube from -15°C to -25°C storage.
 - 4 Remove the adhesive seal from the ALP plate.
 - 5 Do one of the following:
 - If using the in-line control reagent:
 - Dilute the Ligation Control to 1/100 in Resuspension Buffer (For example, $1 \mu\text{l}$ Ligation Control + $99 \mu\text{l}$ Resuspension Buffer) before use. Discard the diluted Ligation Control after use.
 - Add $2.5 \mu\text{l}$ of diluted Ligation Control to each well of the ALP plate.
 - If not using the in-line control reagent, add $2.5 \mu\text{l}$ of Resuspension Buffer to each well of the ALP plate.
 - 6 Add $2.5 \mu\text{l}$ of Ligation Mix to each well of the ALP plate.
 - 7 Return the Ligation Mix tube to -15°C to -25°C storage immediately after use.
 - 8 Do one of the following:
 - If using RNA Adapter tubes, add $2.5 \mu\text{l}$ of the thawed RNA Adapter Index to each well of the ALP plate.
 - If using a RAP:
 - Place the RAP on the benchtop so that the part number barcode, on the long side of the plate, is facing you and the clipped corner is on the lower left.

Figure 9 Correct RAP Orientation



- Do one of the following to pierce the foil seal:
 - If using the entire plate at one time, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously. Gently, but firmly, press the clean plate over the foil seal.
 - If using only part of the plate, use the bottom of a clean eight-tube strip, with caps attached, to pierce holes in the seals of the wells that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each row or column of adapters that will be used for ligation.
 - Using an eight-tip multichannel pipette, transfer 2.5 μ l of the appropriate thawed RNA Adapter from the RAP well to each well of the ALP plate.
- 9 Mix thoroughly as follows:
 - a Seal the ALP plate with a Microseal 'B' adhesive seal.
 - b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.
 - 10 Centrifuge the ALP plate to $280 \times g$ for 1 minute.

Incubate 2 ALP

- 1 Place the sealed ALP plate on the pre-heated microheating system. Close the lid and incubate at 30°C for 10 minutes.
- 2 Remove the ALP plate from the microheating system.

Add STL

- 1 Remove the adhesive seal from the ALP plate.
- 2 Add 5 μ l of Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation mix. Mix thoroughly as follows:
 - a Seal the ALP plate with a Microseal 'B' adhesive seal.
 - b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.
- 3 Centrifuge the ALP plate to $280 \times g$ for 1 minute.

Clean Up ALP

- 1 Remove the adhesive seal from the ALP plate.
- 2 Vortex the AMPure XP Beads for at least 1 minute or until they are well dispersed.

- 3 Add 42 μ l of mixed AMPure XP Beads to each well of the ALP plate. Mix thoroughly as follows:
 - a Seal the ALP plate with a Microseal 'B' adhesive seal.
 - b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.
- 4 Incubate the ALP plate at room temperature for 15 minutes.
- 5 Centrifuge the ALP plate to $280 \times g$ for 1 minute.
- 6 Remove the adhesive seal from the ALP plate.
- 7 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
- 8 Remove and discard 79.5 μ l supernatant from each well of the ALP plate. Take care not to disturb the beads.

**NOTE**

Leave the ALP plate on the magnetic stand while performing the following 80% EtOH wash steps (9–11).

- 9 With the ALP plate on the magnetic stand, add 200 μ l freshly prepared 80% EtOH to each well without disturbing the beads.
- 10 Incubate the ALP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.
- 11 Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.
- 12 With the ALP plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes.
- 13 Remove the ALP plate from the magnetic stand.
- 14 Add 52.5 μ l Resuspension Buffer to each well of the ALP plate. Mix thoroughly as follows:
 - a Seal the ALP plate with a Microseal 'B' adhesive seal.
 - b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.
- 15 Incubate the ALP plate at room temperature for 2 minutes.
- 16 Centrifuge the ALP plate to $280 \times g$ for 1 minute.
- 17 Remove the adhesive seal from the ALP plate.

- 18 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
- 19 Transfer 50 μ l supernatant from each well of the ALP plate to the corresponding well of the new MIDI plate labeled with the CAP barcode. Take care not to disturb the beads.
- 20 Vortex the AMPure XP Beads until they are well dispersed.
- 21 Add 50 μ l of mixed AMPure XP Beads to each well of the CAP plate for a second cleanup. Mix thoroughly as follows:
 - a Seal the CAP plate with a Microseal 'B' adhesive seal.
 - b Shake the CAP plate on a microplate shaker at 1800 rpm for 2 minutes.
- 22 Incubate the CAP plate at room temperature for 15 minutes.
- 23 Centrifuge the CAP plate to $280 \times g$ for 1 minute.
- 24 Remove the adhesive seal from the CAP plate.
- 25 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
- 26 Remove and discard 95 μ l supernatant from each well of the CAP plate. Take care not to disturb the beads.

**NOTE**

Leave the CAP plate on the magnetic stand while performing the following 80% EtOH wash steps (27–29)

- 27 With the CAP plate on the magnetic stand, add 200 μ l freshly prepared 80% EtOH to each well. Take care not to disturb the beads.
- 28 Incubate the CAP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.
- 29 Repeat steps 27 and 28 one time for a total of two 80% EtOH washes.
- 30 With the CAP plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes.
- 31 Remove the CAP plate from the magnetic stand.
- 32 Add 22.5 μ l Resuspension Buffer to each well of the CAP plate. Mix thoroughly as follows:
 - a Seal the CAP plate with a Microseal 'B' adhesive seal.
 - b Shake the CAP plate on a microplate shaker at 1800 rpm for 2 minutes.

- 33 Incubate the CAP plate at room temperature for 2 minutes.
- 34 Centrifuge the CAP plate to $280 \times g$ for 1 minute.
- 35 Remove the adhesive seal from the CAP plate.
- 36 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
- 37 Transfer 20 μ l supernatant from each well of the CAP plate to the corresponding well of the new HSP plate labeled with the PCR barcode. Take care not to disturb the beads.



SAFESTOPPING POINT

If you do not plan to proceed immediately to *Enrich DNA Fragments* on page 81, you can safely stop the protocol here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to seven days.

Enrich DNA Fragments

This process uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the amount of DNA in the library. The PCR is performed with a PCR Primer Cocktail that anneals to the ends of the adapters. Minimize the number of PCR cycles to avoid skewing the representation of the library.



NOTE

PCR enriches for fragments that have adapters ligated on both ends. Fragments with only one or no adapters on their ends are by-products of inefficiencies in the ligation reaction. Neither species can be used to make clusters. Fragments without any adapters cannot hybridize to surface-bound primers in the flow cell. Fragments with an adapter on only one end can hybridize to surface bound primers, but cannot form clusters.

Consumables

Item	Quantity	Storage	Supplied By
PCR Master Mix (PMM)	1 tube per 48 reactions	-15°C to -25°C	Illumina
PCR Primer Cocktail (PPC)	1 tube per 48 reactions	-15°C to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Barcode labels for: <ul style="list-style-type: none"> • CPP (Clean Up PCR Plate) barcode label • TSP1 (Target Sample Plate) barcode label 	1 label per plate	15°C to 30°C	Illumina
96-well HSP Plate	1	15°C to 30°C	User
96-well MIDI Plate	1	15°C to 30°C	User
AMPure XP Beads	50 µl per sample	2°C to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
Microseal 'A' Film	1	15°C to 30°C	User
Microseal 'B' Adhesive Seals	3	15°C to 30°C	User
RNase/DNase-free Eight-Tube Strips and Caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- ▶ Remove the PCR Master Mix and PCR Primer Cocktail from -15°C to -25°C storage and thaw them at room temperature.
- ▶ Centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 × g for 5 seconds.
- ▶ Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- ▶ Remove the AMPure XP Beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- ▶ Remove the PCR plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 77.
 - Let it thaw at room temperature.
 - Centrifuge the thawed PCR plate to 280 × g for 1 minute.
 - Remove the adhesive seal from the thawed PCR plate.

- ▶ Pre-program the thermal cycler with the following program and save as **PCR**:
 - Choose the pre-heat lid option and set to 100°C
 - 98°C for 30 seconds
 - 15 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
- ▶ Apply a CPP barcode label to a new 96-well MIDI plate.
- ▶ Apply a TSP1 barcode label to a new 96-well 0.3 ml PCR plate.

Make PCR

- 1 Add 5 µl of thawed PCR Primer Cocktail to each well of the PCR plate.
- 2 Add 25 µl of thawed PCR Master Mix to each well of the PCR plate.
 - a Seal the PCR plate with a Microseal 'A' film.



WARNING

Follow vendor instructions for applying Microseal "A" sealing films. Improper use could lead to inefficient sealing (evaporation of sample or cross-contamination) or too efficient sealing (parts of the seal remain in the well after removing the whole seal).


- b Shake the PCR plate on a microplate shaker at 1600 rpm for 20 seconds.
- 3 Centrifuge the PCR plate to 280 × g for 1 minute.

Amp PCR

- 1 Place the sealed PCR plate on the pre-programmed thermal cycler. Close the lid, then select and run **PCR** to amplify the plate.
 - a Choose the pre-heat lid option and set to 100°C
 - b 98°C for 30 seconds
 - c 15 cycles of:
 - 98°C for 10 seconds
 - 60°C for 30 seconds
 - 72°C for 30 seconds

- d 72°C for 5 minutes
- e Hold at 4°C

Clean Up PCR

- 1 Remove the adhesive seal from the PCR plate.
 - 2 Vortex the AMPure XP Beads until they are well dispersed.
 - 3 Do one of the following, depending on the adapter type used:
 - If using the RNA Adapter tubes, add 50 μ l of the mixed AMPure XP Beads to each well of the new MIDI plate labeled with the CPP barcode.
 - If using the RAP, add 47.5 μ l of the mixed AMPure XP Beads to each well of the new MIDI plate labeled with the CPP barcode.
 - 4 Transfer the entire contents from each well of the PCR plate to the corresponding well of the CPP plate containing 50 μ l of mixed AMPure XP Beads. Mix thoroughly as follows:
 - a Seal the CPP plate with a Microseal 'B' adhesive seal.
 - b Shake the CPP plate on a microplate shaker at 1800 rpm for 2 minutes.
 - 5 Incubate the CPP plate at room temperature for 15 minutes.
 - 6 Centrifuge the CPP plate to 280 \times g for 1 minute.
 - 7 Remove the adhesive seal from the CPP plate.
 - 8 Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
 - 9 Remove and discard 95 μ l supernatant from each well of the CPP plate.
-  **NOTE**
Leave the CPP plate on the magnetic stand while performing the following 80% EtOH wash steps (10–12).
- 10 With the CPP plate on the magnetic stand, add 200 μ l freshly prepared 80% EtOH to each well without disturbing the beads.
 - 11 Incubate the CPP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.
 - 12 Repeat steps 10 and 11 one time for a total of two 80% EtOH washes.

- 13 With the CPP plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes, and then remove the plate from the magnetic stand.
- 14 Add 32.5 μ l Resuspension Buffer to each well of the CPP plate. Mix thoroughly as follows:
 - a Seal the CPP plate with a Microseal 'B' adhesive seal.
 - b Shake the CPP plate on a microplate shaker at 1800 rpm for 2 minutes.
- 15 Incubate the CPP plate at room temperature for 2 minutes.
- 16 Centrifuge the CPP plate to $280 \times g$ for 1 minute.
- 17 Remove the adhesive seal from the CPP plate.
- 18 Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
- 19 Transfer 30 μ l supernatant from each well of the CPP plate to the corresponding well of the new HSP plate labeled with the TSP1 barcode.

**SAFESTOPPING POINT**

If you do not plan to proceed immediately to *Validate Library* on page 86, you can safely stop the protocol here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C for up to 7 days.

Validate Library

Illumina recommends performing the following procedures for quality control analysis on your sample library and quantification of the DNA library templates.

Quantify Libraries

To achieve the highest quality data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of the flow cell. Optimizing cluster densities requires accurate quantitation of DNA library templates. Quantify your libraries using qPCR according to the Illumina *Sequencing Library qPCR Quantification Guide* (part # 11322363).

Quality Control

- 1 Load 1 μ l of the resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA-specific chip such as the Agilent DNA 1000.
- 2 Check the size and purity of the sample. The final product should be a band at approximately 260 bp.

Figure 10 Example of TruSeq Stranded Total RNA Sample Preparation Library Size Distribution

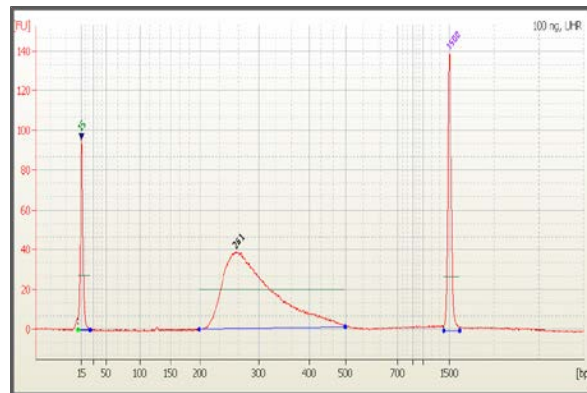
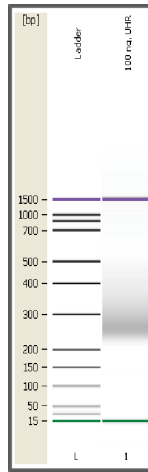


Figure 11 TruSeq Stranded Total RNA Sample Preparation 260 bp PCR Product



Normalize and Pool Libraries

This process describes how to prepare DNA templates for cluster generation. Indexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. DNA libraries not intended for pooling are normalized to 10 nM in the DCT plate.

Consumables

Item	Quantity	Storage	Supplied By
Barcode labels for: <ul style="list-style-type: none"> • DCT (Diluted Cluster Template) • PDP (Pooled DCT Plate) (for pooling only) 	1 label per plate	15°C to 30°C	Illumina
96-well HSP Plate (for pooling only)	1	15°C to 30°C	User
96-well MIDI Plate	1	15°C to 30°C	User
Microseal 'B' Adhesive Seals	5	15°C to 30°C	User
Tris-HCl 10 mM, pH8.5 with 0.1% Tween 20	Enough to normalize the concentration of each sample library to 10 nM	15°C to 30°C	User

Preparation

- ▶ Remove the TSP1 plate from -15°C to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 84.
 - Let it thaw at room temperature.
 - Centrifuge the thawed TSP1 plate to 280 × g for 1 minute.
 - Remove the adhesive seal from the thawed TSP1 plate.
- ▶ Apply a DCT barcode label to a new 96-well MIDI plate.
- ▶ [For pooling only] Apply a PDP barcode label to a new 96-well HSP plate.

Make DCT

- 1 Transfer 10 μ l of sample library from each well of the TSP1 plate to the corresponding well of the new MIDI plate labeled with the DCT barcode.
- 2 Normalize the concentration of sample library in each well of the DCT plate to 10 nM using Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20.



NOTE

Depending on the yield quantification data of each sample library, the final volume in the DCT plate can vary from 10–400 μ l.

- 3 Mix the DCT plate as follows:
 - a Seal the DCT plate with a Microseal 'B' adhesive seal.
 - b Shake the DCT plate on a microplate shaker at 1000 rpm for 2 minutes.
- 4 Centrifuge the DCT plate to $280 \times g$ for 1 minute.
- 5 Remove the adhesive seal from the DCT plate.
- 6 Depending on the type of library you want to generate, do one of the following:
 - For non-pooled libraries, the protocol stops here. Do one of the following:
 - Proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina platform.
 - Seal the DCT plate with a Microseal 'B' adhesive seal and store at -15°C to -25°C .
 - For pooled libraries, proceed to *Make PDP (for pooling only)*.

Make PDP (for pooling only)



NOTE

Do not make a PDP plate if you are not pooling samples.

- 1 Determine the number of samples to be combined together for each pool.



NOTE

Make a note of which sample goes into which well, to avoid pooling two samples with the same index.

- 2 Do one of the following:
 - If pooling 2–24 samples:
 - Transfer 10 μ l of each normalized sample library to be pooled from the DCT plate to one well of the new HSP plate labeled with the PDP barcode.
 - The total volume in each well of the PDP plate should be 10X the number of combined sample libraries and 20–240 μ l (2–24 libraries). For example, the volume for 2 samples is 20 μ l, the volume for 12 samples is 120 μ l, or the volume for 24 samples is 240 μ l.
 - If pooling 25–96 samples:
 - Using a multichannel pipette, transfer 5 μ l of each normalized sample library in column 1 from the DCT plate to column 1 of the new HSP plate labeled with the PDP barcode.
 - Transfer 5 μ l of each normalized sample library in column 2 from the DCT plate to column 1 of the PDP plate.
 - Repeat the transfer for as many times as there are remaining columns in the DCT plate. The result is a PDP plate with pooled samples in column 1. Mix the PDP plate as follows:
 - Seal the PDP plate with a Microseal 'B' adhesive seal.
 - Shake the PDP plate on a microplate shaker at 1800 rpm for 2 minutes.
 - Centrifuge the PDP plate to $280 \times g$ for 1 minute.
 - Remove the adhesive seal from the PDP plate.
 - Combine the contents of each well of column 1 into well A2 of the PDP plate for the final pool.
- 3 Mix the PDP plate as follows:
 - a Seal the PDP plate with a Microseal 'B' adhesive seal.
 - b Shake the PDP plate on a microplate shaker at 1800 rpm for 2 minutes.
- 4 Centrifuge the PDP plate to $280 \times g$ for 1 minute.
- 5 Do one of the following:
 - Proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina platform.
 - Store the sealed PDP plate at -15°C to -25°C .

Supporting Information

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Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed your kit contents, and obtained all of the requisite consumables and equipment.

Acronyms

Table 9 TruSeq Stranded Total RNA Sample Preparation Acronyms

Acronym	Definition
ALP	Adapter Ligation Plate
ATL	A-Tailing Mix
BRP	Bind rRNA Plate
CAP	Clean Up ALP Plate
CCP	cDNA Clean Up Plate
cDNA	Complementary DNA
CPP	Clean Up PCR Plate
CTA	A-Tailing Control
CTE	End Repair Control
CTL	Ligation Control
DCT	Diluted Cluster Template
DFP	Depleted RNA Fragmentation Plate
ds cDNA	Double-Stranded Complimentary DNA
ELB	Elution Buffer
EPH	Elute, Prime, Fragment High Mix
EUC	Experienced User Card
FFPE	Formalin-Fixed, Paraffin-Embedded
FSA	First Strand Synthesis Act D Mix

Acronym	Definition
GRM	Globin Removal Mix
H/M/R	Human/Mouse/Rat
HS	High Sample
HSP	Hardshell Plate
HT	High Throughput
IEM	Illumina Experiment Manager
LIG	Ligation Mix
LS	Low Sample
LT	Low Throughput
LTF	Lab Tracking Form
PCR	Polymerase Chain Reaction
PDP	Pooled Dilution Plate
PMM	PCR Master Mix
PPC	PCR Primer Cocktail
RAP	RNA Adapter Plate
RBB	rRNA Binding Buffer
RCP	RNA CleanUp Plate
RRB	rRNA Removal Beads
RRM	rRNA Removal Mix
RRM G	rRNA Removal Mix - Gold
RRM P	rRNA Removal Mix - Plant

Acronym	Definition
rRNA	Ribosomal RNA
RRP	rRNA Removal Plate
RSB	Resuspension Buffer
SMM	Second Strand Marking Master Mix
STL	Stop Ligation Buffer
TSP	Target Sample Plate

Kit Contents

Check to make sure that you have all of the reagents identified in this section before starting the TruSeq Stranded Total RNA Sample Preparation protocol. The TruSeq Stranded Total RNA LT Sample Prep Kits are available as Set A and B. Each TruSeq Stranded Total RNA LT Sample Prep Kit contains enough reagents to prepare up to 24 samples. When used together, TruSeq Stranded Total RNA LT Sample Prep Kits A and B allow for pooling up to 24 samples using the 12 different indices in each kit.

Table 10 TruSeq Stranded Total RNA Sample Preparation Kits

Kit Name	Catalog #	Number of Samples Supported	Number of Indices
TruSeq Stranded Total RNA LT Sample Prep Kit - Set A (with Ribo-Zero Human/Mouse/Rat)	RS-122-2201	48	12
TruSeq Stranded Total RNA LT Sample Prep Kit - Set B (with Ribo-Zero Human/Mouse/Rat)	RS-122-2202	48	12
TruSeq Stranded Total RNA HT Sample Prep Kit (with Ribo-Zero Human/Mouse/Rat)	RS-122-2203	96	96
TruSeq Stranded Total RNA LT Sample Prep Kit - Set A (with Ribo-Zero Gold)	RS-122-2301	48	12
TruSeq Stranded Total RNA LT Sample Prep Kit - Set B (with Ribo-Zero Gold)	RS-122-2302	48	12

Kit Name	Catalog #	Number of Samples Supported	Number of Indices
TruSeq Stranded Total RNA HT Sample Prep Kit (with Ribo-Zero Gold)	RS-122-2303	96	96
TruSeq Stranded Total RNA LT Sample Prep Kit - Set A (with Ribo-Zero Plant)	RS-122-2401	48	12
TruSeq Stranded Total RNA LT Sample Prep Kit - Set B (with Ribo-Zero Plant)	RS-122-2402	48	12
TruSeq Stranded Total RNA HT Sample Prep Kit (with Ribo-Zero Plant)	RS-122-2403	96	96
TruSeq Stranded Total RNA LT Sample Prep Kit - Set A (with Ribo-Zero Globin)	RS-122-2501	48	12
TruSeq Stranded Total RNA LT Sample Prep Kit - Set B (with Ribo-Zero Globin)	RS-122-2502	48	12
TruSeq Stranded Total RNA HT Sample Prep Kit (with Ribo-Zero Globin)	RS-122-2503	96	96

TruSeq Stranded Total RNA LT Sample Prep Kit

The TruSeq Stranded Total RNA LT Sample Prep Kit contains four boxes: an A or B box, Box 1, Box 2, and a cDNA Synthesis PCR box.

48 Samples, 12 Index Set A and B

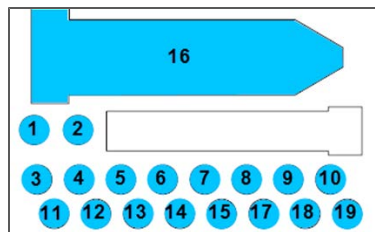
You receive either box A or B in the kit, depending on the set ordered.

Store at -15°C to -25°C

These boxes are shipped on dry ice. As soon as you receive your kit, store the following components at -15°C to -25°C.

Set A

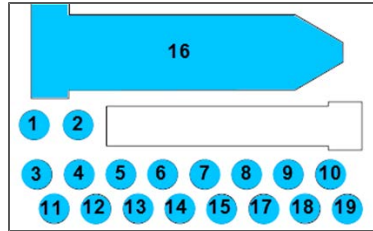
Figure 12 TruSeq Stranded Total RNA LT Sample Prep Kit 48 Samples, 12 Index Set A, part # 15032612



Slot	Reagent	Part #	Description
1	LIG	15026773	Ligation Mix
2	ATL	15012495	A-Tailing Mix
3	STL	15012546	Stop Ligation Buffer
4	AR013	15024655	RNA Adapter Index 13
5	AR014	15024656	RNA Adapter Index 14
6	AR015	15024657	RNA Adapter Index 15
7	AR016	15024658	RNA Adapter Index 16
8	AR018	15024660	RNA Adapter Index 18
9	AR019	15024661	RNA Adapter Index 19
10	AR002	15026634	RNA Adapter Index 2
11	AR004	15026636	RNA Adapter Index 4
12	AR005	15026637	RNA Adapter Index 5
13	AR006	15026638	RNA Adapter Index 6
14	AR007	15026640	RNA Adapter Index 7
15	AR012	15026645	RNA Adapter Index 12
16	RSB	15026770	Resuspension Buffer
17	CTE	15026774	End Repair Control
18	CTA	15026775	A-Tailing Control
19	CTL	15026776	Ligation Control

Set B

Figure 13 TruSeq Stranded Total RNA LT Sample Prep Kit 48 Samples, 12 Index Set B, part # 15032613



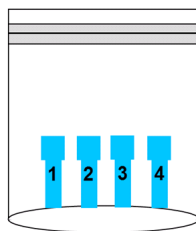
Slot	Reagent	Part #	Description
1	ATL	15012495	A-Tailing Mix
2	STL	15012546	Stop Ligation Buffer
3	AR020	15024662	RNA Adapter Index 20
4	AR021	15024663	RNA Adapter Index 21
5	AR022	15024664	RNA Adapter Index 22
6	AR023	15024665	RNA Adapter Index 23
7	AR025	15024667	RNA Adapter Index 25
8	AR027	15024668	RNA Adapter Index 27
9	AR001	15026633	RNA Adapter Index 1
10	AR003	15026635	RNA Adapter Index 3
11	AR008	15026641	RNA Adapter Index 8
12	AR009	15026642	RNA Adapter Index 9
13	AR010	15026643	RNA Adapter Index 10
14	AR011	15026644	RNA Adapter Index 11
15	RSB	15026770	Resuspension Buffer
16	LIG	15026773	Ligation Mix
17	CTE	15026774	End Repair Control
18	CTA	15026775	A-Tailing Control
19	CTL	15026776	Ligation Control

48 Samples, Box 1 of 2

Store as specified

This box is shipped on refrigerated gel packs. As soon as you receive it, store the following components as specified.

Figure 14 TruSeq Stranded Total RNA LT Sample Prep Kit, 48 Samples (Box 1 of 2), part # 15032615



Slot	Reagent	Part #	Description	Storage Temperature
1	DTE	15026766	CTE Dilution Tube	Room Temperature
2	DTA	15026805	CTA Dilution Tube	Room Temperature
3	DTL	15026807	CTL Dilution Tube	Room Temperature
4	RRB	15031727	rRNA Removal Beads	2°C to 8°C

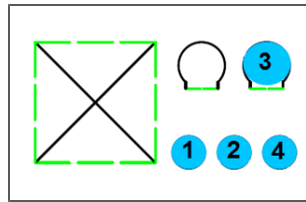
48 Samples Ribo-Zero Box

You will receive one of the following boxes, depending on the kit ordered. These boxes also contain plate barcode labels.

Store as specified

This box is shipped on dry ice. As soon as you receive it, store the following components as specified.

Figure 15 TruSeq Stranded Total RNA LT Sample Prep Kit 48 Samples Ribo-Zero



Ribo-Zero H/M/R, part # 15032618

Slot	Reagent	Part #	Description	Storage Temperature
1	RBB	15031737	rRNA Binding Buffer	-15°C to -25°C
2	RRM	15031738	rRNA Removal Mix	-15°C to -25°C
3	ELB	15026780	Elution Buffer	2°C to 8°C
4	EPH	15029211	Elute, Prime, Fragment High Mix	-15°C to -25°C

Ribo-Zero Gold, part # 15032619

Slot	Reagent	Part #	Description	Storage Temperature
1	RBB	15031737	rRNA Binding Buffer	-15°C to -25°C
2	RRM G	15033133	rRNA Removal Mix - Gold	-15°C to -25°C
3	ELB	15026780	Elution Buffer	2°C to 8°C
4	EPH	15029211	Elute, Prime, Fragment High Mix	-15°C to -25°C

Ribo-Zero Plant, part # 15035748

Slot	Reagent	Part #	Description	Storage Temperature
1	RBB	15031737	rRNA Binding Buffer	-15°C to -25°C
2	RRM P	15033135	rRNA Removal Mix - Plant	-15°C to -25°C
3	ELB	15026780	Elution Buffer	2°C to 8°C
4	EPH	15029211	Elute, Prime, Fragment High Mix	-15°C to -25°C

Ribo-Zero Globin, part # 15035750

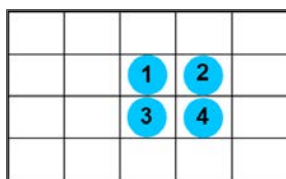
Slot	Reagent	Part #	Description	Storage Temperature
1	RBB	15031737	rRNA Binding Buffer	-15°C to -25°C
2	GRM	15037137	Globin Removal Mix	-15°C to -25°C
3	ELB	15026780	Elution Buffer	2°C to 8°C
4	EPH	15029211	Elute, Prime, Fragment High Mix	-15°C to -25°C

48 Samples, cDNA Synthesis PCR Box

Store at -15°C to -25°C

This box is shipped on dry ice. As soon as you receive it, store the following components at -15°C to -25°C.

Figure 16 TruSeq Stranded Total RNA LT Sample Prep Kit, 48 Samples, cDNA Synthesis PCR Box, part # 15032611



Slot	Reagent	Part #	Description
1	PMM	15026785	PCR Master Mix
2	PPC	15031748	PCR Primer Cocktail
3	FSA	15031094	First Strand Synthesis Act D Mix
4	SMM	15031098	Second Strand Marking Master Mix

TruSeq Stranded Total RNA HT Sample Prep Kit

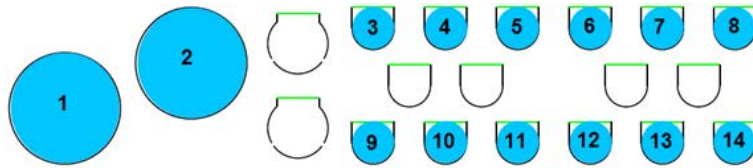
The TruSeq Stranded Total RNA HT Sample Prep Kit contains five boxes: a core reagent box, a cDNA Synthesis- PCR box, an Adapter Plate box, and a Box 1 and Box 2.

96 Samples, Core Box

Store at -15°C to -25°C

This box is shipped on dry ice. As soon as you receive it, store the following components at -15°C to -25°C.

Figure 17 TruSeq Stranded Total RNA HT Sample Prep Kit, 96 Samples, Core Box, part # 15032620



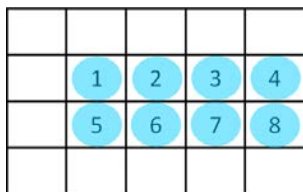
Slot	Reagent	Part #	Description
1-2	RSB	15026770	Resuspension Buffer
3-4	ATL	15012495	A-Tailing Mix
5-6	LIG	15026773	Ligation Mix
7-8	CTE	15026774	End Repair Control
9-10	CTA	15026775	A-Tailing Control
11-12	CTL	15026776	Ligation Control
13-14	STL	15012546	Stop Ligation Buffer

96 Samples, cDNA Synthesis-PCR Box

Store at -15°C to -25°C

This box is shipped on dry ice. As soon as you receive it, store the following components at -15°C to -25°C.

Figure 18 TruSeq Stranded Total RNA HT Sample Prep Kit, 96 Samples, cDNA Synthesis-PCR Box, part # 15032621



Slot	Reagent	Part #	Description
1-2	PMM	15026785	PCR Master Mix
3-4	PPC	15031748	PCR Primer Cocktail
5-6	FSA	15031094	First Strand Synthesis Act D Mix
7-8	SMM	15031098	Second Strand Marking Master Mix

96 Samples- Adapter Plate Box

Store at -15°C to -25°C

This box is shipped on dry ice. As soon as you receive it, store the contents at -15°C to -25°C.

Figure 19 TruSeq Stranded Total RNA HT Sample Prep Kit, 96, Adapter Plate Box, part # 15032622



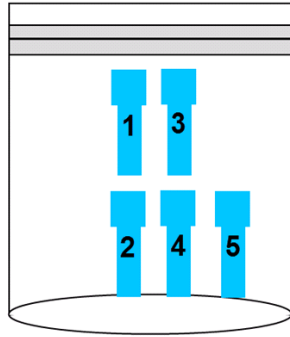
Slot	Reagent	Part #	Description
1	RAP	15016427	RNA Adapter Plate, 96plex

96 Samples, Box 1 of 2

Store as specified

This box is shipped on refrigerated gel packs. As soon as you receive it, store the following components as specified.

Figure 20 TruSeq Stranded Total RNA HT Sample Prep Kit, 96 Samples (Box 1 of 2), part # 15032625



Slot	Reagent	Part #	Description	Storage Temperature
1-2	RRB	15031727	rRNA Removal Beads	2°C to 8°C
3	DTL	15026807	CTL Dilution Tube	Room Temperature
4	DTE	15026766	CTE Dilution Tube	Room Temperature
5	DTA	15026805	CTA Dilution Tube	Room Temperature

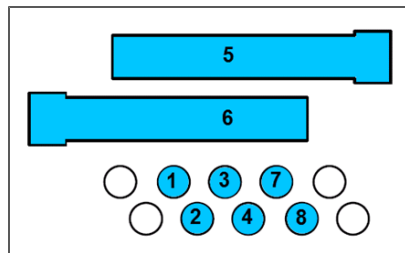
96 Samples Ribo-Zero Box

You will receive one of the following boxes, depending on the kit ordered. These boxes also contain plate barcode labels.

Store as specified

This box is shipped on dry ice. As soon as you receive it, store the following components as specified.

Figure 21 TruSeq Stranded Total RNA HT Sample Prep Kit, 96 Samples Ribo-Zero



Ribo-Zero H/M/R, part # 15032626

Slot	Reagent	Part #	Description	Storage Temperature
1-2	RBB	15031737	rRNA Binding Buffer	-15°C to -25°C
3-4	RRM	15031738	rRNA Removal Mix	-15°C to -25°C
5-6	ELB	15026780	Elution Buffer	2°C to 8°C
7-8	EPH	15029211	Elute, Prime, Fragment High Mix	-15°C to -25°C

Ribo-Zero Gold, part # 15032627

Slot	Reagent	Part #	Description	Storage Temperature
1-2	RBB	15031737	rRNA Binding Buffer	-15°C to -25°C
3-4	RRM G	15033133	rRNA Removal Mix - Gold	-15°C to -25°C
5-6	ELB	15026780	Elution Buffer	2°C to 8°C
7-8	EPH	15029211	Elute, Prime, Fragment High Mix	-15°C to -25°C

Ribo-Zero Plant, part # 15035749

Slot	Reagent	Part #	Description	Storage Temperature
1-2	RBB	15031737	rRNA Binding Buffer	-15°C to -25°C
3-4	RRM P	15033135	rRNA Removal Mix - Plant	-15°C to -25°C
5-6	ELB	15026780	Elution Buffer	2°C to 8°C
7-8	EPH	15029211	Elute, Prime, Fragment High Mix	-15°C to -25°C

Ribo-Zero Globin, part # 15035751

Slot	Reagent	Part #	Description	Storage Temperature
1-2	RBB	15031737	rRNA Binding Buffer	-15°C to -25°C
3-4	GRM	15037137	Globin Removal Mix	-15°C to -25°C
5-6	ELB	15026780	Elution Buffer	2°C to 8°C
7-8	EPH	15029211	Elute, Prime, Fragment High Mix	-15°C to -25°C

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before starting the TruSeq Stranded Total RNA Sample Preparation protocol. The requirement for some supplies is dependent upon the protocol performed (LS or HS) and these items are specified in separate tables.

Table 11 User-Supplied Consumables

Consumable	Supplier
1.5 ml RNase/DNase-free non-sticky tubes	Life Technologies, part # AM12450
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
1000 µl single channel 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 storage plates, round well, 0.8 ml ("MIDI" plate)	Fisher Scientific, part # AB-0859
96-well 2 ml deep well plates (Optional - to aliquot reagents)	Thomson Instrument Company, part # 951652
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881

Consumable	Supplier
Agencourt RNAClean XP 40 ml kit	Beckman Coulter Genomics, part # A63987
Agilent RNA 6000 Nano Kit or Agilent RNA 6000 Pico Kit (Optional - for alternative fragmentation only)	Agilent Technologies, part # 5067-1511 or part # 5067-1513
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
MicroTube (6x16mm), AFA fiber with crimp-cap (Optional - for alternative fragmentation only)	Covaris, part # 520052
MinElute Gel Extraction Kit (Optional - if starting with previously isolated mRNA)	QIAGEN, part # 28604
Nuclease-free ultra pure water	General lab supplier
RNaseZap (to decontaminate surfaces)	General lab supplier
RNase/DNase-free eight-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
SuperScript II Reverse Transcriptase	Invitrogen, part # 18064-014
Tris-HCl 10 mM, pH8.5	General lab supplier
Tween 20	Sigma, part # P7949

Table 12 User-Supplied Consumables - Additional Items for LS Processing

Consumable	Supplier
96-well 0.3 ml PCR plates	General lab supplier

Table 13 User-Supplied Consumables - Additional Items for HS Processing

Consumable	Supplier
Microseal 96-well PCR plates ("HSP" plate)	Bio-Rad, part # HSP-9601
Microseal 'A' film	Bio-Rad, part # MSA-5001

Table 14 User-Supplied Equipment

Equipment	Supplier
96-well thermal cycler (with heated lid)	General lab supplier
2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
Agilent DNA 1000 Kit	Agilent, part # 5067-1504
Magnetic stand-96	Life Technologies, part # AM10027
Microplate centrifuge	General lab supplier
Vortexer	General lab supplier

Table 15 User-Supplied Equipment - Additional Items for HS Processing

Consumable	Supplier
High-Speed Microplate Shaker	VWR, catalog # <ul style="list-style-type: none"> • 13500-890 (110 V/120 V) or • 14216-214 (230 V)
MIDI plate insert for heating system Note: Two inserts are recommended to support successive heating procedures.	Illumina, catalog # BD-60-601
Stroboscope	General lab supplier

Consumable	Supplier
<p>One of the following: Note: Two systems are recommended to support successive heating procedures.</p> <ul style="list-style-type: none"><li data-bbox="389 370 778 394">• SciGene TruTemp Heating System<li data-bbox="389 472 738 496">• Hybex Microsample Incubator	<ul style="list-style-type: none"><li data-bbox="986 375 1246 467">• Illumina, catalog #<ul style="list-style-type: none"><li data-bbox="1007 407 1246 431">• SC-60-503 (115 V) or<li data-bbox="1007 440 1214 464">• SC-60-504 (220 V)<li data-bbox="986 480 1241 573">• SciGene, catalog #<ul style="list-style-type: none"><li data-bbox="1007 513 1241 537">• 1057-30-0 (115 V) or<li data-bbox="1007 545 1209 570">• 1057-30-2 (230 V)

Indexed Adapter Sequences

This section details the indexed adapter sequences.

TruSeq Stranded Total RNA LT Sample Prep Kit Indexed Adapter Sequences

The TruSeq Stranded Total RNA LT Sample Prep Kit contains the following indexed adapter sequences.



NOTE

- The index numbering is not contiguous. There is no Index 17, 24, or 26.
- The base in parentheses () indicates the base for the seventh cycle and is not considered as part of the index sequence. Record the index in the sample sheet as only six bases. For indices 13 and above, the seventh base (in parentheses) might not be A, which is seen in the seventh cycle of the index read.
- For more information on the number of cycles used to sequence the index read, reference your instrument user guide.

Table 16 TruSeq Stranded Total RNA LT Sample Prep Kit Set A Indexed Adapter Sequences

Adapter	Sequence	Adapter	Sequence
AR002	CGATGT(A)	AR013	AGTCAA(C)
AR004	TGACCA(A)	AR014	AGTTCC(G)
AR005	ACAGTG(A)	AR015	ATGTCA(G)
AR006	GCCAAT(A)	AR016	CCGTCC(C)
AR007	CAGATC(A)	AR018	GTCCGC(A)
AR012	CTTGTA(A)	AR019	GTGAAA(C)

Table 17 TruSeq Stranded Total RNA LT Sample Prep Kit Set B Indexed Adapter Sequences

Adapter	Sequence	Adapter	Sequence
AR001	ATCACG(A)	AR020	GTGGCC(T)
AR003	TTAGGC(A)	AR021	GTTTCG(G)
AR008	ACTTGA(A)	AR022	CGTACG(T)
AR009	GATCAG(A)	AR023	GAGTGG(A)
AR010	TAGCTT(A)	AR025	ACTGAT(A)
AR011	GGCTAC(A)	AR027	ATTCCT(T)

TruSeq Stranded Total RNA HT Sample Prep Kit Indexed Adapter Sequences

The RAP in the TruSeq Stranded Total RNA HT Sample Prep Kit contains the following indexed adapter sequences:



NOTE

The Index recorded in the sample sheet is the full 8 bases and 8 bases are sequenced per indexed read.

Table 18 TruSeq Stranded Total RNA HT Sample Prep Kit Indexed Adapter 1 Sequences

Adapter	Sequence	Adapter	Sequence
D701	ATTACTCG	D707	CTGAAGCT
D702	TCCGGAGA	D708	TAATGCGC
D703	CGCTCATT	D709	CGGCTATG
D704	GAGATTCC	D710	TCCGCGAA
D705	ATTCAGAA	D711	TCTCGCGC
D706	GAATTCGT	D712	AGCGATAG

Table 19 TruSeq Stranded Total RNA HT Sample Prep Kit Indexed Adapter 2 Sequences

Adapter	Sequence	Adapter	Sequence
D501	TATAGCCT	D505	AGGCGAAG
D502	ATAGAGGC	D506	TAATCTTA
D503	CCTATCCT	D507	CAGGACGT
D504	GGCTCTGA	D508	GTACTGAC

Alternate Fragmentation Protocols

Introduction	116
Modify RNA Fragmentation Time for Intact RNA	117
Modify RNA Fragmentation Time for Degraded RNA	119



Introduction

Fragmentation of the nucleic acids is required for optimal library preparation, clustering, and sequencing. When starting with intact RNA, the TruSeq Stranded Total RNA fragmentation protocol for transcriptome analysis is performed on the RNA after rRNA depletion using elevated temperatures. This results in libraries with inserts ranging in size from 120–200 bp with a median size of 150 bp. The TruSeq Stranded Total RNA fragmentation protocol ensures the best coverage of the transcriptome with efficient library production.

Illumina recognizes that some customers have different purposes for their sequencing experiments. The need for larger inserts is greater than the need for the best coverage for applications such as splice variant analysis studies. To vary the insert size of your library, see *Modify RNA Fragmentation Time for Intact RNA* on page 117.

Illumina also recognizes that it is not always possible to extract intact total RNA. For instance, RNA extracted from FFPE samples is typically degraded. To vary the fragmentation time for degraded RNA, see *Modify RNA Fragmentation Time for Degraded RNA* on page 119.

Modify RNA Fragmentation Time for Intact RNA

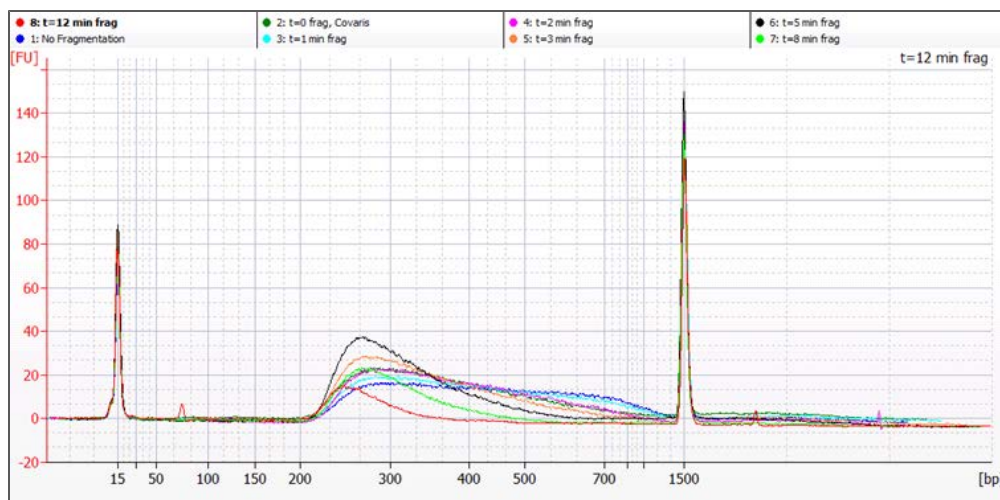
To modify the fragmentation of the RNA to allow for longer RNA fragments, the time of fragmentation can be shortened. This is accomplished during the *Ribo-Zero Deplete and Fragment RNA* procedures by modifying the thermal cycler **Elution 2 - Frag - Prime** program: 94°C for X minutes followed by a 4°C hold for the thermal cycler. X is determined by the length of RNA desired. A range of suggested times and sizes is described in Table 20.

Table 20 Library Insert Fragmentation Time

Time at 94°C (minutes)	Range of Insert Length ^a (bp)	Median Insert Length ^a (bp)	Average Final Library Size (Bioanalyzer bp)
0 ^b	130–350	200	467
1	130–310	190	439
2	130–290	185	410
3	125–250	165	366
4	120–225	160	326
8	120–210	155	309
12	115–180	140	272

- a. Insert length determined after clustering, and sequencing with a paired-end sequencing run.
 b. Instead of a 94°C incubation, incubate at 65°C for 5 minutes, followed by a 4°C hold. This will elute the mRNA from the beads without fragmentation. The resulting cDNA fragments are smaller than the mRNA due to internal priming by the random hexamers in the EPH.

Figure 22 Shortened Fragmentation Time Results



NOTE

The discrepancy between the reported insert size using the Agilent Bioanalyzer and the insert size determined after clustering, and sequencing with a paired-end sequencing run is due to the bias towards clustering smaller fragments. To target a specific fragment size, a gel size selection step is required after adapter ligation.

Modify RNA Fragmentation Time for Degraded RNA

For degraded RNA samples, the fragmentation time must be adjusted to avoid over fragmentation of the RNA samples. This is accomplished during the *Ribo-Zero Deplete and Fragment RNA* procedures by either skipping fragmentation (*Incubate 1 DFP*) or modifying the thermal cycler **Elution 2 - Frag - Prime** program to 94°C for X minutes, followed by a 4°C hold.

Whether or not the samples should undergo fragmentation and the amount of time used for fragmentation (X) is determined by the size range of the total RNA starting material. To determine which fragmentation settings to use, if any:

- 1 Measure the size range of the total RNA starting material by running it on a Agilent RNA 6000 Nano or Pico chip.
- 2 Compare the resulting electropherogram to Figure 23–Figure 27, which show UHR that has been fragmented to various size ranges.
- 3 Determine which sample figure most resembles the size range of your starting material.
- 4 Use the thermal cycler settings recommended in the figure title of that size range to fragment your degraded RNA samples.

For starting material smaller than that shown in Figure 27, no fragmentation is necessary. Skip *Incubate 1 DFP* and proceed immediately to *Synthesize First Strand cDNA*.

Figure 23 Incubate Samples at 94°C for 8 Minutes, Followed By a 4°C Hold

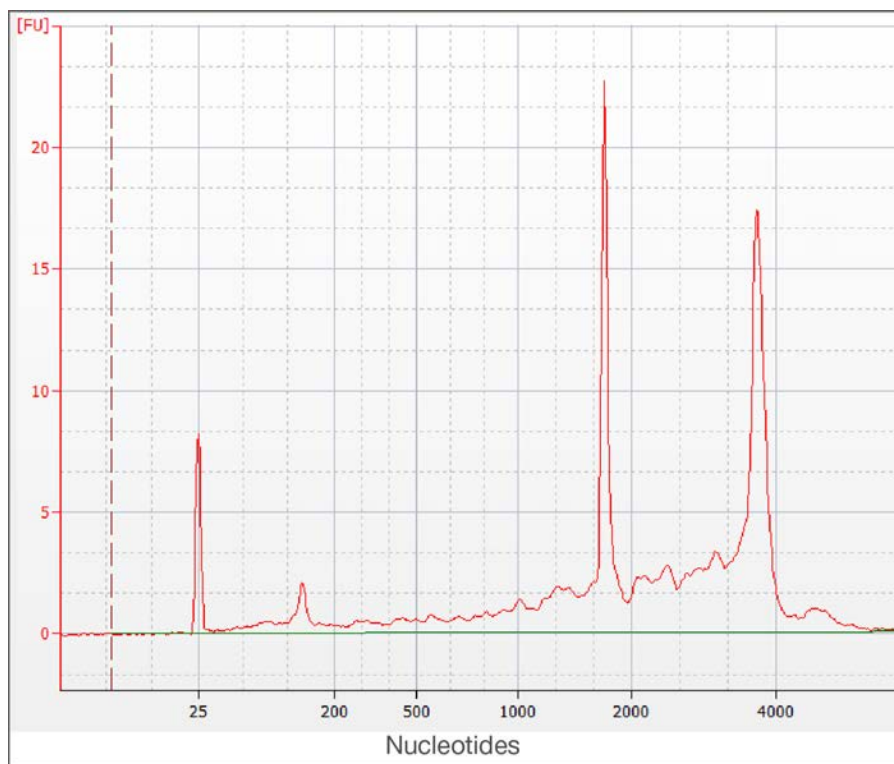


Figure 24 Incubate Samples at 94°C for 7 Minutes, Followed By a 4°C Hold

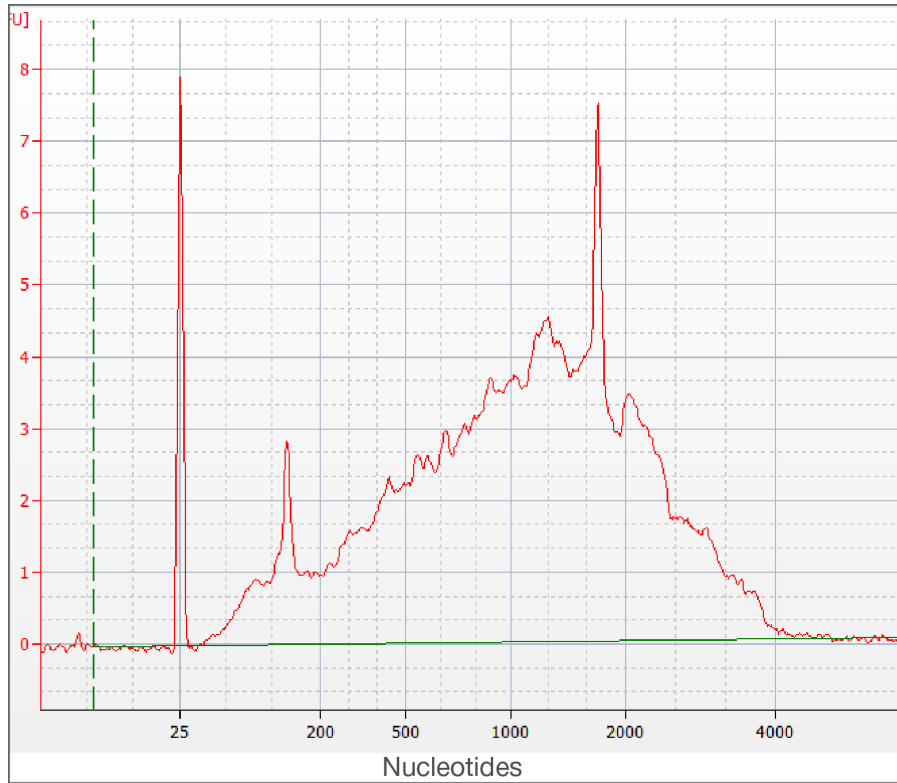


Figure 25 Incubate Samples at 94°C for 6 Minutes, Followed By a 4°C Hold

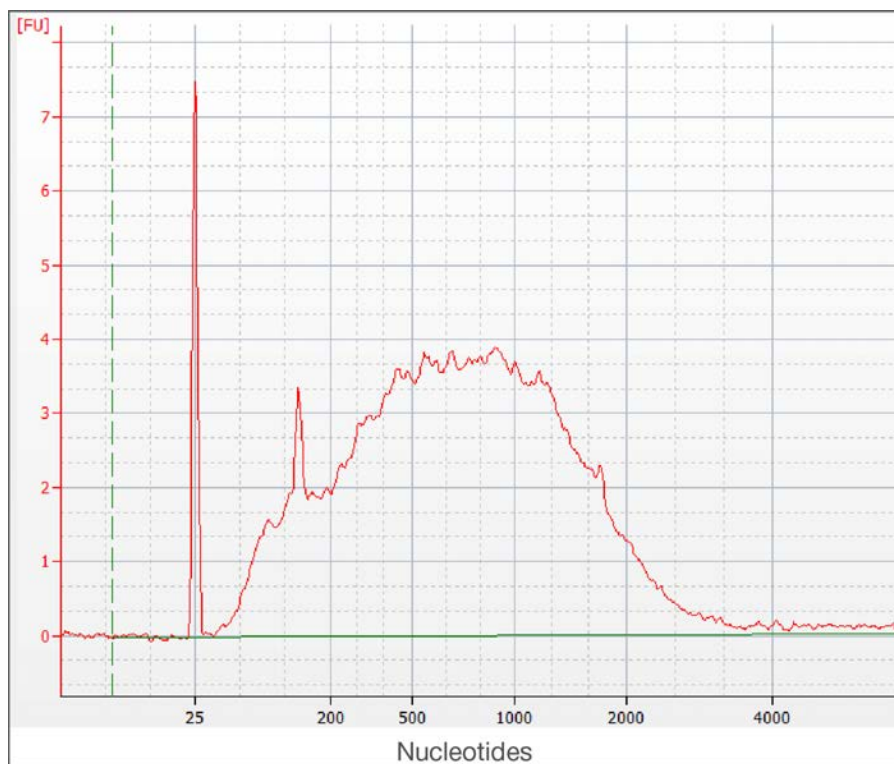


Figure 26 Incubate Samples at 94°C for 4 Minutes, Followed By a 4°C Hold

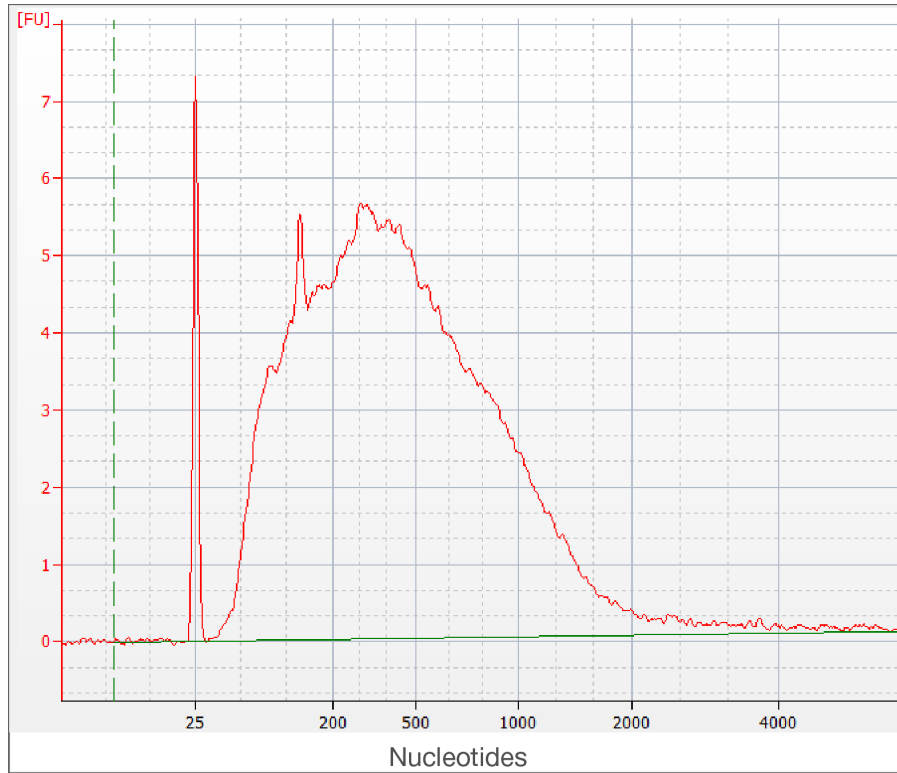
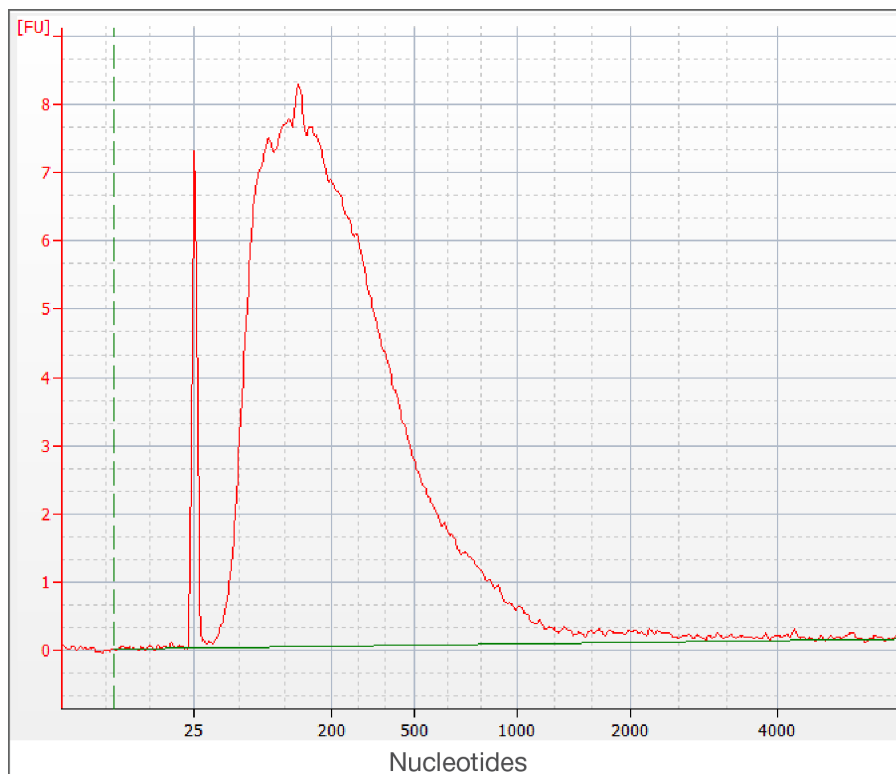


Figure 27 No Fragmentation Necessary. Skip *Incubate 1 DFP* and Proceed to *Synthesize First Strand cDNA*



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Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 21 Illumina General Contact Information

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

Table 22 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at www.illumina.com/msds.

Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support, select a product, then click **Documentation & Literature**.

