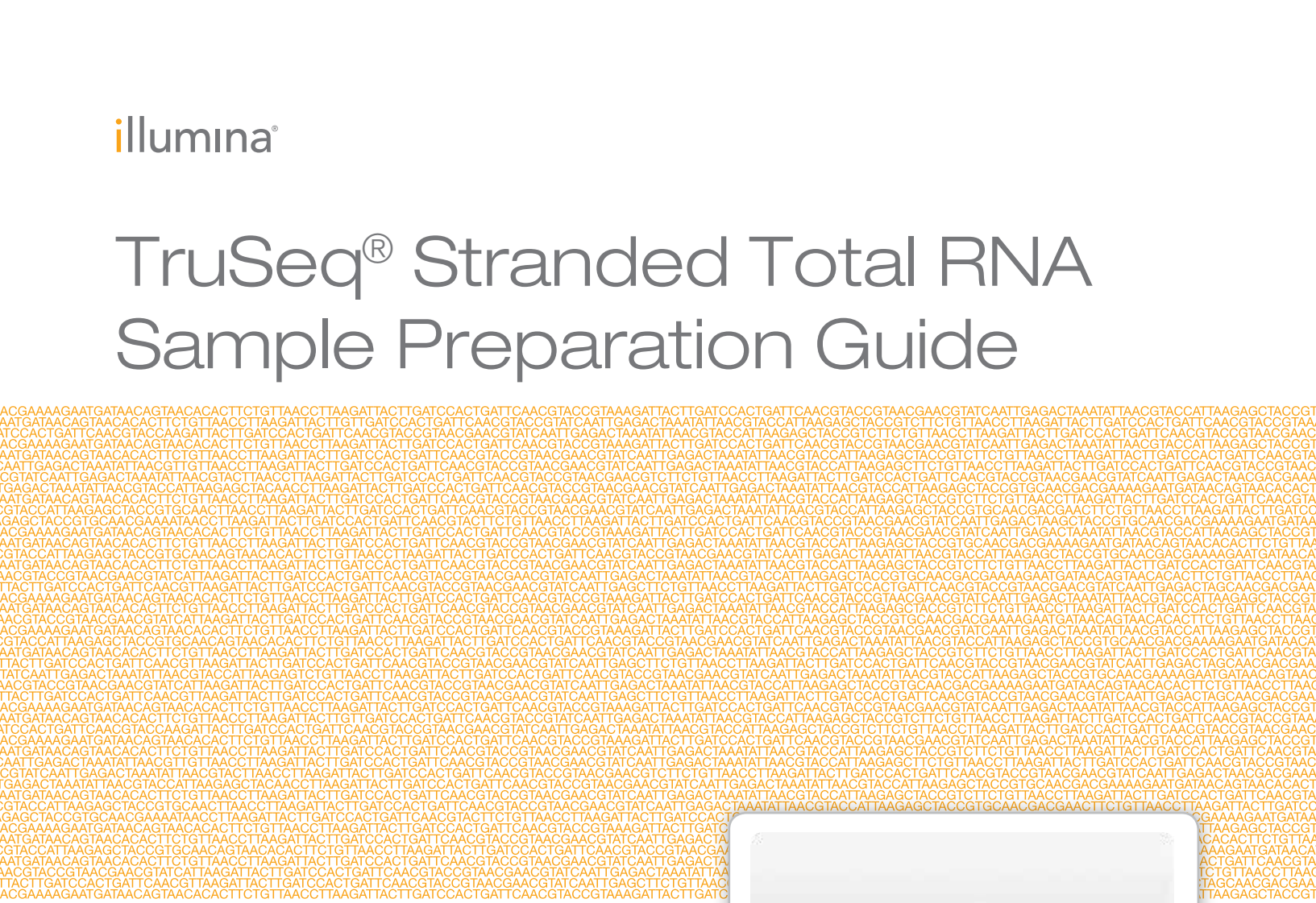
illumina®

TruSeq® Stranded Total RNA Sample Preparation Guide



FOR RESEARCH USE ONLY

ILLUMINA PROPRIETARY

Part # 15031048 Rev. C
September 2012

Revision History

Part #	Revision	Date	Description of Change
15031048	C	September 2012	• 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° to 8°C storage
15031048	B	July 2012	• AddedTruSeq Stranded Total RNA HT Sample Prep Kitcontent and functionality to the following sections: • *Usage Guidelines* • *Kit Contents* • *Indexed Adapter Sequences* • *Adapter Options* • *Pooling Guidelines* • *Ligate Adapters*procedures • *Enrich DNA Fragments*procedures • *Normalize and Pool Libraries s*procedures • Added reagent volume table to*Usage Guidelines* • Revised*Tracking Tools*documentation download information • Removed detailed Sample Sheet description from*Tracking Tools* • 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*Consumables and Equipment*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

Table of Contents

Revision History iii
Table of Contents v
List of Tables vii

Chapter 1 Overview 1

Introduction 2
Audience and Purpose 4

Chapter 2 Getting Started 7

Introduction 8
Acronyms 9
Best Practices 12
RNA Input Recommendations 25
In-Line Control DNA 27
Tracking Tools 29
Kit Contents 30
Consumables and Equipment 40
Indexed Adapter Sequences 43
Adapter Options 46
Pooling Guidelines 51

Chapter 3 Low Sample (LS) Protocol 61

Introduction 62
Sample Prep Workflow 64
Ribo-Zero Deplete and Fragment RNA 65
Synthesize First Strand cDNA 73
Synthesize Second Strand cDNA 76
Adenylate 3' Ends 80
Ligate Adapters 82

Enrich DNA Fragments 89
Validate Library 93
Normalize and Pool Libraries 95

Chapter 4 High Sample (HS) Protocol 99

Introduction 100
Sample Prep Workflow 102
Ribo-Zero Deplete and Fragment RNA 103
Synthesize First Strand cDNA 112
Synthesize Second Strand cDNA 115
Adenylate 3' Ends 119
Ligate Adapters 122
Enrich DNA Fragments 129
Validate Library 134
Normalize and Pool Libraries 136

Appendix A Alternate Fragmentation Protocols 139

Introduction 140
Modify RNA Fragmentation Time for Intact RNA 141
Modify RNA Fragmentation Time for Degraded RNA 143

Index 149

Technical Assistance 153

List of Tables

Table 1 Protocol Features 4
Table 2 Kit and Sample Number Recommendations 4
Table 3 Kit and Protocol Recommendations 5
Table 4 TruSeq Stranded Total RNA Sample Preparation Acronyms 9
Table 5 TruSeq Stranded Total RNA Sample Prep Reagent Volumes 16
Table 6 In-Line Control Functions 27
Table 7 TruSeq Stranded Total RNA Sample Preparation Kits 30
Table 8 Ribo-Zero Human/Mouse/Rat, part # 15032618 34
Table 9 Ribo-Zero Gold, part # 15032619 34
Table 10 Ribo-Zero Human/Mouse/Rat, part # 15032626 39
Table 11 Ribo-Zero Gold, part # 15032627 39
Table 12 User-Supplied Consumables 40
Table 13 User-Supplied Consumables - Additional Items for LS Processing 41
Table 14 User-Supplied Consumables - Additional Items for HS Processing 42
Table 15 User-Supplied Equipment 42
Table 16 User-Supplied Equipment - Additional Items for HS Processing 42
Table 17 TruSeq Stranded Total RNA LT Sample Prep Kit Indexed Adapter Sequences 43
Table 18 TruSeq Stranded Total RNA HT Sample Prep Kit Indexed Adapter Sequences 44
Table 19 Dual-Indexed Sequencing Platform Compatibility 47
Table 20 Single-Indexed Pooling Strategies for 2–4 Samples 51
Table 21 Kit and Sample Number Recommendations 62
Table 22 Kit and Protocol Recommendations 62
Table 23 Kit and Sample Number Recommendations 100
Table 24 Kit and Protocol Recommendations 100
Table 25 Library Insert Fragmentation Time 141
Table 26 Illumina General Contact Information 153
Table 27 Illumina Customer Support Telephone Numbers 153

Overview

Chapter 1

Introduction 2
Audience and Purpose 4

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.

The TruSeq Stranded Total RNA kits with Ribo-Zero™ Human/Mouse/Rat and the TruSeq Stranded Total RNA kits with Ribo-Zero Gold both support human, mouse, and rat organisms and follow the same workflow. The first step involves the removal of ribosomal RNA using Ribo-Zero rRNA removal beads. The Ribo-Zero Human/Mouse/Rat depletes samples of cytoplasmic ribosomal RNA and the Ribo-Zero Gold depletes samples of both cytoplasmic and mitochondrial ribosomal RNA. 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. This is 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.

Audience and Purpose

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 3 Low Sample (LS) Protocol explains how to perform the TruSeq Stranded Total RNA Sample Preparation using the Low Sample (LS) Protocol
- Chapter 4 High Sample (HS) Protocol explains how to perform the TruSeq Stranded Total RNA Sample Preparation using the High Sample (HS) 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	Micro plate 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	Kit Recommended
<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

Getting Started

Chapter 2

Introduction 8
Acronyms 9
Best Practices 12
RNA Input Recommendations 25
In-Line Control DNA 27
Tracking Tools 29
Kit Contents 30
Consumables and Equipment 40
Indexed Adapter Sequences 43
Adapter Options 46
Pooling Guidelines 51

Introduction

This chapter explains standard operating procedures and precautions for performing TruSeq Stranded Total RNA Sample Preparation. You will also find lists of standard equipment and consumables.

The sample preparation protocols described in the rest of this guide assume that you are familiar with the contents of this chapter, have implemented all the recommendations, and have obtained all of the requisite equipment and consumables.

Acronyms

Table 4 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 Mix Act D

Acronym	Definition
HSP	Hardshell Plate
HS	High Sample
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
RIP	Removal Intermediate Plate
RPB	RNA Purification Beads
RRB	rRNA Removal Beads
RRM	rRNA Removal Mix
RRP	rRNA Removal Plate

Acronym	Definition
RSB	Resuspension Buffer
SIP	Second Removal Intermediate Plate
SMM	Second Strand Marking Master Mix
STL	Stop Ligation Buffer
TSP	Target Sample Plate

Best Practices

When preparing RNA libraries for sequencing, you should always adhere to good molecular biology practices. Read through the entire protocol prior to starting to ensure all of the required materials are available and your equipment is programmed and ready to use.

NOTE

For more information, see the *TruSeq Sample Preparation Best Practices and Troubleshooting Guide* which you can download from the Illumina website at http://www.illumina.com. Go to the TruSeq Stranded Total RNA Sample Preparation support page and click the **Documentation & Literature** tab. A MyIllumina account is required.

Handling RNA

RNA is highly susceptible to degradation by RNAse enzymes. RNAse enzymes are present in cells and tissues and carried on hands, labware, and dust. They are very stable and difficult to inactivate. For these reasons, it is important to follow best laboratory practices while preparing and handling RNA samples.

- When harvesting total RNA, use a method that quickly disrupts tissue and isolates and stabilizes RNA.
- Wear gloves and use sterile technique at all times.
- Reserve a set of pipettes for RNA work. Use sterile RNAse-free filter pipette tips to prevent cross-contamination.
- Use disposable plasticware that is certified to be RNAse-free. Illumina recommends the use of non-stick sterile RNAse-free microfuge tubes. A set of these tubes should be designated for this protocol and should not be used for other lab work.
- All reagents should be prepared from RNAse-free components, including ultra pure water.
- Store RNA samples by freezing. Avoid extended pauses in the protocol until the RNA is in the form of double-stranded cDNA (ds cDNA).
- Use a RNAse/DNAse decontamination solution to decontaminate work surfaces and equipment prior to starting this protocol.

Handling Liquids

Good liquid handling measures are essential, particularly when quantifying libraries or diluting concentrated libraries for making clusters.

- Small differences in volumes (±0.5 µl) can sometimes give rise to very large differences in cluster numbers (~100,000).
- Small volume pipetting can be a source of potential error in protocols that require generation of standard curves, such as PicoGreen assays or qPCR, or those that require small but precise volumes, such as the Agilent Bioanalyzer.
- If small volumes are unavoidable, then due diligence should be taken to make sure that pipettes are correctly calibrated.
- Make sure that pipettes are not used at the volume extremes of their performance specifications.

Handling Master Mix Reagents

When handling the master mix reagents:

- Minimize freeze-thaw cycles. If you do not intend to consume the reagents in one use, dispense the reagent into aliquots after the initial thaw and refreeze the aliquots in order to avoid excessive freeze-thaw cycles. However, if you aliquot, you might not have enough reagents for the full number of reactions over multiple uses.
- Add reagents in the order indicated and avoid making master-mixes containing the in-line controls.
- Take care while adding the A-Tailing Mix (ATL) and Ligation Mix (LIG) due to the viscosity of the reagents.
- First Strand Synthesis Mix Act D (FSA) 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. Please 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 http://www.illumina.com/msds.

Handling Magnetic Beads

Follow appropriate handling methods when working AMPure XP and RNAClean XP Beads:

NOTE
Cleanup procedures have only been validated using the 96-well plates and the magnetic stand specified in the Consumables and Equipment list. Comparable performance is not guaranteed when using a microcentrifuge tube or other formats, or other magnets.

- Prior to use, allow the beads to come to room temperature.
- Do not reuse beads. Always add fresh beads when performing these procedures.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous.
- **When performing the LS protocol**:
  - After adding the beads to the reaction, mix the solution gently and thoroughly by pipetting up and down 10 times, making sure the liquid comes in contact with the beads and that the beads are resuspended homogeneously.
  - Pipetting with the tips at the bottom of the well and not pipetting the entire volume of the solution helps prevent the solution from foaming. Excessive foaming leads to sample loss, because the foam is not transferred out of the plate efficiently.
- **When performing the HS protocol**, after adding the beads to the reaction, seal the plate and shake the plate on a microplate shaker at 1,800 rpm for 2 minutes. Repeat, if necessary, until the color of the mixture appears homogeneous after mixing.
- Take care to minimize bead loss which can impact final yields.
- Change the tips for each sample.
- Let the mixed samples incubate for 15 minutes at room temperature for maximum recovery.
- When aspirating the cleared solution from the reaction plate and wash step, it is important to keep the plate on the magnetic stand and to not disturb the separated magnetic beads. Aspirate slowly to prevent the beads from sliding down the sides of the wells and into the pipette tips.
- To prevent the carryover of beads after elution, approximately 2.5 µl of supernatant are left when the eluates are removed from the bead pellet.

- Prepare fresh 70% and 80% ethanol. Ethanol tends to absorb water from the air, therefore, fresh 70% and 80% ethanol should be prepared for optimal results.
- Be sure to remove all of the ethanol from the bottom of the wells, as it can contain residual contaminants.
- Keep the reaction plate on the magnetic stand and let it air-dry at room temperature to prevent potential bead loss due to electrostatic forces. Allow for the complete evaporation of residual ethanol, as the presence of ethanol will impact the performance of the subsequent reactions. Illumina recommends at least 15 minutes drying time, but a longer drying time might be required. Remaining ethanol can be removed with a 20 µl pipette.
- Use the Elution Buffer (ELB) for RNA elution.
- Avoid over drying the beads, which can impact final yields.
- **When performing the LS protocol**, resuspend the dried pellets using a single channel or multichannel pipette.
- **When performing the HS protocol**, resuspend the dried pellets by shaking.
- When removing and discarding supernatant from the wells, use a single channel or multichannel pipette and take care not to disturb the beads.
- To maximize sample recovery during elution, incubate the sample/bead mix for 2 minutes at room temperature before placing the samples onto the magnet.

Avoiding Cross-Contamination

Practice the following to avoid cross-contamination:

- Open only one adapter tube at a time.
- Clean the bottom of the 96-well PCR plate or eight-tube strip used to pierce the foil seal of a RNA Adapter Plate (RAP) with a sterile 70% Ethanol wipe.
- Pipette carefully to avoid spillage.
- Clean pipettes and change gloves between handling different adapter stocks.
- Clean work surfaces thoroughly before and after the procedure.

Temperature Considerations

Temperature is an important consideration for making cDNA libraries:

- Keep libraries at temperatures ≤37°C, except where specifically noted.
- Place reagents on ice after thawing at room temperature.

- When processing more than 48 samples manually, Illumina recommends processing the plate on a bed of ice whenever possible, especially during the enzymatic steps (when using the A-Tailing Mix and Ligation Mix). A large number of samples processed at room temperature may result in uneven catalytic activity, which can lead to reduced quality of the end product.
- mRNA fragments that have a high AT content are more likely to denature into single strands than GC-rich fragments, which can result in an increased probability of creating a bias in the sequencing coverage.
- Temperature is less of an issue after the adapters have been ligated onto the ends of the ds cDNA.

Usage Guidelines

Illumina recommends these guidelines as the most efficient lab setup and pipetting process when performing the procedures specified in Chapter 3 Low Sample (LS) Protocol and Chapter 4 High Sample (HS) Protocol.

NOTE
The TruSeq Stranded Total RNA LT Sample Prep Kit contains enough of each reagent to prepare 48 samples at one time and the TruSeq Stranded Total RNA HT Sample Prep Kit contains enough reagent to prepare 96 samples at one time. If an alternate lab setup and pipetting process is used, Illumina cannot guarantee that there will be enough of every reagent for the full number of samples.

NOTE
When using multichannel pipettes, take care to pipette accurately into the wells, as variations in volume will affect the sample preparation. Change tips after each sample.

Reference the following table to determine the required reagent volume per sample for these guidelines.

Table 5 TruSeq Stranded Total RNA Sample Prep Reagent Volumes

Reagent	Description	Volume per Sample (µl)
AR0XX or RAP	RNA Adapter tube or RNA Adapter Plate	2.5
ATL	A-Tailing Mix	12.5
CTA	A-Tailing Control	2.5 of 1/100 dilution

Reagent	Description	Volume per Sample (µl)
CTE	End Repair Control	5 of 1/50 dilution
CTL	Ligation Control	2.5 of 1/100 dilution
ELB	Elution Buffer	11
EPH	Elute, Prime, Fragment High Mix	8.5
FSA	First Strand Synthesis Act D Mix	8
LIG	Ligation Mix	2.5
PMM	PCR Master Mix	25
PPC	PCR Primer Cocktail	5
RBB	rRNA Binding Buffer	5
RRB	rRNA Removal Beads	35
RRM or RRM G	Ribo-Zero rRNA Removal Mix or Ribo-Zero rRNA Removal Mix Gold	5
SMM	Second Strand Marking Master Mix	20
STL	Stop Ligation Buffer	5

Preparing More Than 24 Samples

When preparing more than 24 samples, follow these guidelines as you perform each procedure in the protocol. Use a multichannel pipette with eight tips to perform all transfers from the reagent vessel to the sample plate.

Sample Distribution

Distribute each sample into a separate column of the plate. Use the appropriate plate for the protocol being performed:

- LS protocol - 96-well 0.3 ml PCR plate
- HS protocol - 96-well MIDI plate and 96-well HSP plate

NOTE

Illumina highly recommends using the Illumina Experiment Manager and reviewing the low-plex pooling guidelines in the *Normalize and Pool Libraries* procedures when setting up the sample plate for use with RAP. Prepare each sample in the sample plate position that corresponds to the desired dual-indexed RNA adapter position in the RAP.

Reagents in Reservoirs

When each of the following reagents is required in the protocol, distribute each into a separate multichannel reagent reservoir as follows:

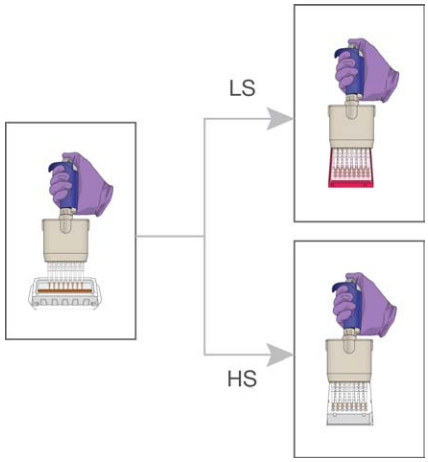
- 70% Ethanol
- 80% Ethanol
- AMPure XP Beads
- Elution Buffer
- Resuspension Buffer
- RNAClean XP Beads

1. Determine the volume needed for each of the above reagents using the equation (# of samples x volume per sample) + 600 µl dead volume. Reference Table 5 or the protocol for the required reagent volume per sample.
2. Fill a separate multichannel reagent reservoir with the determined amount of each reagent.

When each of the above reagents is required in the protocol, distribute each to the sample plate as follows:

1. Using an eight tip multichannel pipette, transfer the reagent in the reservoir to the samples in the plate as follows, while holding the pipette vertically. Reference Table 5 or the protocol for the required reagent volume per sample.

Figure 1 Transfer Reagent from Reservoir to Sample Plate with 24 or More Samples



a Pipette the required reagent volume per sample from the reservoir.
b Add the reagent to column 1 of the sample plate. Change the tips.
c Pipette the required reagent volume per sample from the reservoir.
d Add the reagent to column 2 of the sample plate. Change the tips.
e Repeat as needed for each column containing a sample.

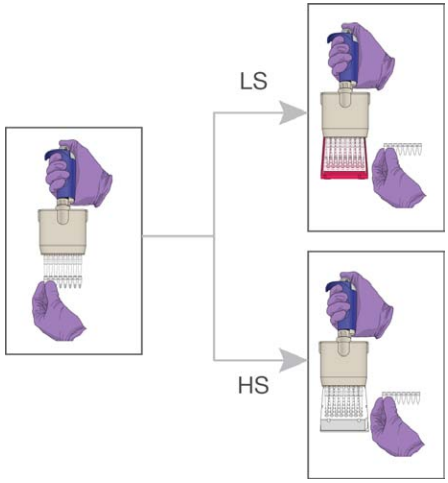
Reagents in Strip Tubes

When the remaining reagents listed in Table 5, except the adapters, are required in the protocol, distribute each evenly across eight wells of an eight-tube strip. Add an allowance of 5 µl for dead volume per well.

When each reagent in an eight-tube strip is required in the protocol, distribute each to the sample plate as follows:

1 Using an eight tip multichannel pipette, transfer the reagent in the eight-tube strip to the samples in the plate as follows, while holding the pipette vertically. Reference Table 5 or the protocol for the required reagent volume per sample.

Figure 2 Transfer Reagent from Strip Tube to Sample Plate with 24 or More Samples



a Pipette the reagent from the eight strip wells.
b Add the reagent to column 1 of the sample plate. Change the tips.
c Pipette the reagent from the eight strip wells.
d Add the reagent to column 2 of the sample plate. Change the tips.
e Repeat as needed for each column containing a sample.

Index Adapters

When using RNA index adapter tubes, do one of the following:

- Add 2.5 µl of the appropriate/desired index adapter individually to each well of the plate containing a sample, using a single channel pipette.
- Using an eight-tube strip:
  - Distribute the index adapters into the wells of an eight-tube strip, with a different adapter in each well.
  - Add 2.5 µl of the appropriate/desired index adapter from the well of the eight-tube strip to each well of the plate containing a sample, using a multichannel pipette.

When using a RAP, see *Handling Adapter Plate* on page 48.

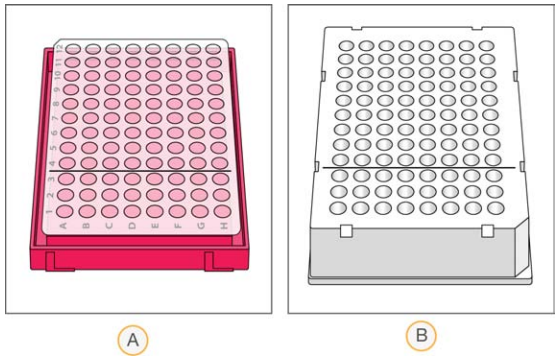
Preparing 12–24 Samples

When preparing 12–24 samples, follow these guidelines as you perform each procedure in the protocol. Use a multichannel pipette with three tips to perform all transfers from the reagent vessel to the sample plate.

Sample Distribution

Distribute the 12–24 samples into three columns and four to eight rows (e.g., four rows per 12 samples) of the plate. Draw a line on the plate to visually separate the three columns. Use the appropriate plate for the protocol being performed:

Figure 3 Draw Line on Plate



A 96-well 0.3 ml PCR plate (LS Protocol)
B 96-well MIDI plate and 96-well HSP plate (HS Protocol)

Reagents in Reservoirs

When each of the following reagents is required in the protocol, distribute each into a separate multichannel reagent reservoir as follows:

- 70% Ethanol
- 80% Ethanol
- AMPure XP Beads
- Resuspension Buffer
- RNAClean XP Beads

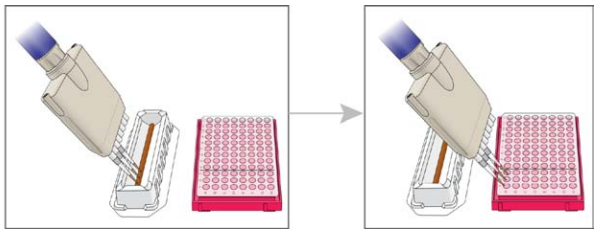
1 Determine the volume needed using the equation (# of samples x volume per sample) + 600 µl dead volume. Reference Table 5 or the protocol for the required reagent volume per sample.

2 Fill a separate multichannel reagent reservoir with the determined amount of each reagent.

When each of the above reagents is required in the protocol, distribute each to the sample plate as follows:

1 Using a multichannel pipette with three tips, transfer the reagent in the reservoir to the samples in the plate as follows, while holding the pipette vertically. Reference Table 5 or the protocol for the required reagent volume per sample.

Figure 4 Transfer Reagent from Reservoir to Sample Plate with 12–24 Samples



a Pipette the required reagent volume per sample from the reservoir.
b Add the reagent to row 1 of the sample plate. Change the tips.
c Pipette the required reagent volume per sample from the reservoir.
d Add the reagent to row 2 of the sample plate. Change the tips.
e Repeat as needed for each row containing a sample.

Reagents in Deep Well Plates

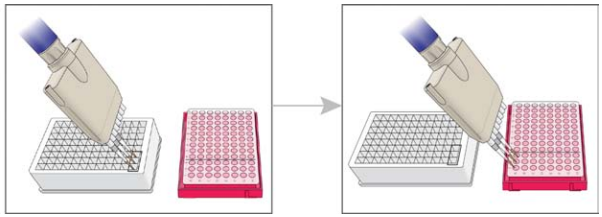
When each of the following reagents are required in the protocol, distribute each into three wells, in the same row of a deep well plate, adding 75 µl dead volume.

- Elution Buffer

When each of the above reagents is required in the protocol, distribute each to the sample plate as follows:

1 Using a multichannel pipette with three tips, transfer the reagent in the deep well plate to the samples in the plate as follows, holding the pipette vertically. Reference Table 5 or the protocol for the required reagent volume per sample.

Figure 5 Transfer Reagent from Deep Well Plate to Sample Plate with 12–24 Samples



a Pipette the reagent from the deep well plate.
b Add the reagent to row 1 of the sample plate. Change the tips.
c Pipette the reagent from the deep well plate.
d Add the reagent to row 2 of the sample plate. Change the tips.
e Repeat as needed for each row containing a sample.

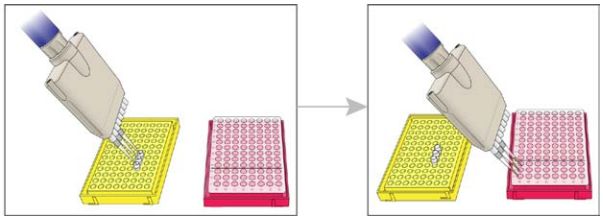
Reagents in Strip Tubes

When the remaining reagents not mentioned above, except the adapters, are required in the protocol, distribute each evenly across the three wells of an eight-tube strip. Add an allowance of 5 µl for dead volume per well.

When each reagent in an eight-tube strip is required in the protocol, distribute each to the sample plate as follows:

1 Using an multichannel pipette with three tips, transfer the reagent in the eight-tube strip to the samples in the plate as follows, while holding the pipette vertically. Reference Table 5 or the protocol for the required reagent volume per sample.

Figure 6 Transfer Reagent from Strip Tube to Sample Plate with 12–24 Samples



a Pipette the reagent from the three strip wells.
b Add the reagent to row 1 of the sample plate. Change the tips.
c Pipette the reagent from the three strip wells.
d Add the reagent to row 2 of the sample plate. Change the tips.

e Repeat as needed for each row containing a sample.

Index Adapter Tubes

When RNA index adapter tubes are used, add 2.5 µl of the appropriate/desired index adapter individually to each well of the plate containing a sample, using a single channel pipette.

Preparing Less Than 12 Samples

When preparing less than 12 samples, follow these guidelines as you perform each procedure in the protocol:

- Add each reagent individually to the samples using a single channel pipette.
- If planning more than three freeze-thaw cycles, aliquot the reagents equally into six separate vessels.

Equipment

Review the programming instructions for your thermal cycler user guide to ensure that it is programmed appropriately using the heated lid function.

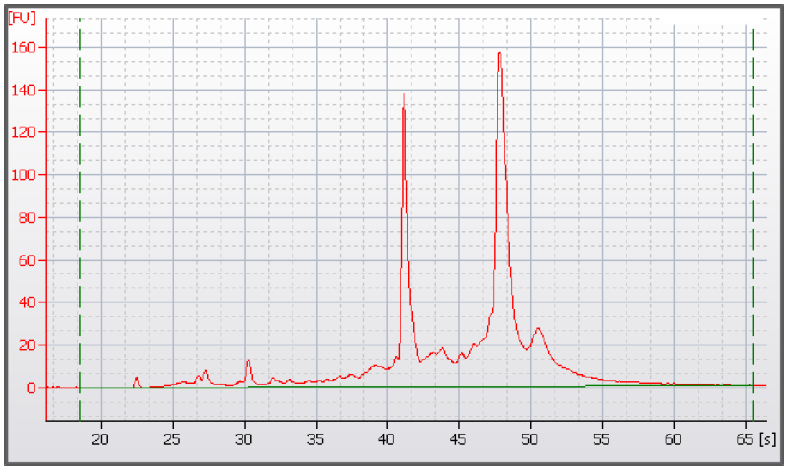
RNA Input Recommendations

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 with regard to 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 very important to know the quality of the RNA starting material. The fragmentation conditions were optimized for high quality RNA.
  - Using the same fragmentation conditions for degraded RNAs, which are shorter in length than full length RNA, 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 7 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. Readout is determined by sequencing. If the sequence of an in-line control appears 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 appears 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 is 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 just prior to 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 6 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 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

Reagent	Function	Control	Structure of Control DNA Ends
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 adapters to 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 a variety of 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 (version 1.9 and higher) recognizes these sequences and isolates the control sequences from the main body of sequencing reads and reports their 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*.

Tracking Tools

Illumina provides the following tools for sample tracking and guidance in the lab:

- **Experienced User Card (EUC)** to guide you through the protocol, but with less detail than provided in this user guide. **New or less experienced users are strongly advised to follow this user guide and not the EUC.**
- **Lab Tracking Form (LTF)** to record information about library preparation such as operator name, sample and index information, start and stop times, reagent lot numbers, and barcodes.
  - Create a copy of the lab tracking form for each time you perform this protocol to prepare a library for sequencing.
  - Use it online and save it electronically or print it and fill it out manually.

NOTE
You can download the above TruSeq Stranded Total RNA Sample Preparation documents from the Illumina website at http://www.illumina.com. Go to the TruSeq Stranded Total RNA Sample Preparation support page and click the **Documentation & Literature** tab. A MyIllumina account is required.

- **Illumina Experiment Manager (IEM)** to create your sample sheet using a wizard-based application. The sample sheet is used to record information about your samples for later use in data analysis. The IEM guides you through the steps to create your sample sheet based on the analysis workflow for your run. The IEM provides a feature for recording parameters for your sample plate, such as sample ID, dual indices, and other parameters applicable to your 96-well plate.
  - When prompted to select a Sample Prep Kit in IEM, choose:
    - **TruSeq LT** if you are using the TruSeq Stranded Total RNA LT Sample Prep Kit
    - **TruSeq HT** if you are using the TruSeq Stranded Total RNA HT Sample Prep Kit

NOTE
IEM can be run on any Windows platform. You can download it from the Illumina website at http://www.illumina.com. A MyIllumina account is required.

Kit Contents

Check to make sure that you have all of the reagents identified in this section before proceeding.

Table 7 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
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

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

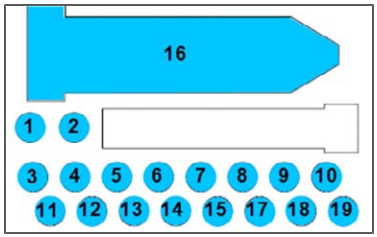
You will receive either box A or B in the kit, depending on the set ordered.

Store at -15° to -25°C

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

Set A

Figure 8 TruSeq Stranded Total RNA LT Sample Prep Kit 48 Samples, 12 Index Set A, part # 15032612

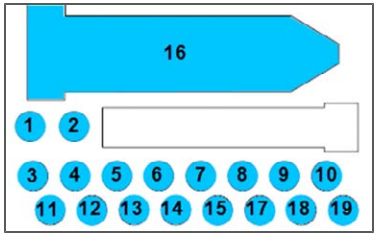


Slot	Reagent	Part #	Description
1	LIG	15026773	Ligation Mix
2	ATL	15012495	A-Tailing Mix
3	STL	15012546	Stop Ligation Buffer
4	AR013	15024655	RNA Adapter Index 13
5	AR014	15024656	RNA Adapter Index 14
6	AR015	15024657	RNA Adapter Index 15
7	AR016	15024658	RNA Adapter Index 16
8	AR018	15024660	RNA Adapter Index 18
9	AR019	15024661	RNA Adapter Index 19

Slot	Reagent	Part #	Description
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 9 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

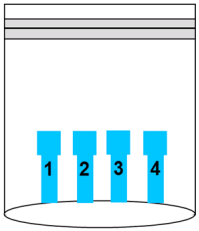
Slot	Reagent	Part #	Description
11	AR008	15026641	RNA Adapter Index 8
12	AR009	15026642	RNA Adapter Index 9
13	AR010	15026643	RNA Adapter Index 10
14	AR011	15026644	RNA Adapter Index 11
15	RSB	15026770	Resuspension Buffer
16	LIG	15026773	Ligation Mix
17	CTE	15026774	End Repair Control
18	CTA	15026775	A-Tailing Control
19	CTL	15026776	Ligation Control

48 Samples, Box 1 of 2

Store as specified

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

Figure 10 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° to 8°C

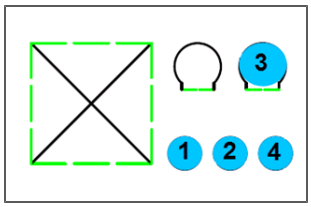
48 Samples, Box 2 of 2

You will receive either a Ribo-Zero Human/Mouse/Rat box 2 or Ribo-Zero Gold box 2, 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 components as specified below.

Figure 11 TruSeq Stranded Total RNA LT Sample Prep Kit 48 Samples (Box 2 of 2)



Ribo-Zero H/M/R Box

Table 8 Ribo-Zero Human/Mouse/Rat, part # 15032618

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

Ribo-Zero Gold Box

Table 9 Ribo-Zero Gold, part # 15032619

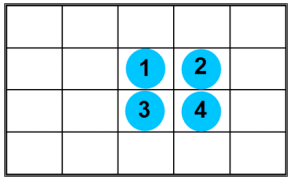
Slot	Reagent	Part #	Description	Storage Temperature
1	RBB	15031737	rRNA Binding Buffer	2° to 8°C
2	RRM G	15033133	rRNA Removal Mix - Gold	-15° to -25°C
3	ELB	15026780	Elution Buffer	2° to 8°C
4	EPH	15029211	Elute, Prime, Fragment High Mix	-15° to -25°C

48 Samples, cDNA Synthesis PCR Box

Store at -15° to -25°C

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

Figure 12 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

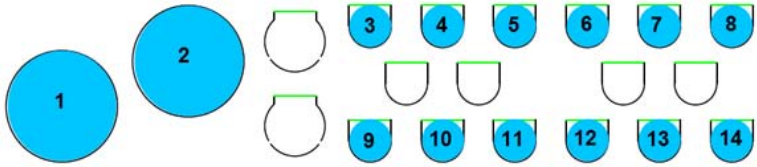
The TruSeq Stranded Total RNA HT Sample Prep Kit contains five boxes: a core reagent box, a cDNA Synthesis- PCR box, an Adapter Plate box, and a Box 1 and Box 2.

96 Samples, Core Box

Store at -15° to -25°C

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

Figure 13 TruSeq Stranded Total RNA HT Sample Prep Kit, 96 Samples, Core Box, part # 15032620



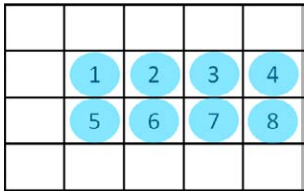
Slot	Reagent	Part #	Description
1–2	RSB	15026770	Resuspension Buffer
3–4	ATL	15012495	A-Tailing Mix
5–6	LIG	15026773	Ligation Mix
7–8	CTE	15026774	End Repair Control
9–10	CTA	15026775	A-Tailing Control
11–12	CTL	15026776	Ligation Control
13–14	STL	15012546	Stop Ligation Buffer

96 Samples, cDNA Synthesis-PCR Box

Store at -15° to -25°C

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

Figure 14 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° to -25°C

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

Figure 15 TruSeq Stranded Total RNA HT Sample Prep Kit, 96 Samples - Adapter Plate Box, part # 15032622

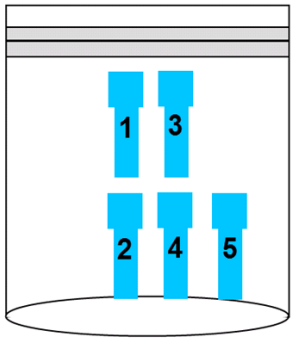
Slot	Reagent	Part #	Description
1	RAP	15016427	RNA Adapter Plate, 96plex

96 Samples, Box 1 of 2

Store as specified

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

Figure 16 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° 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

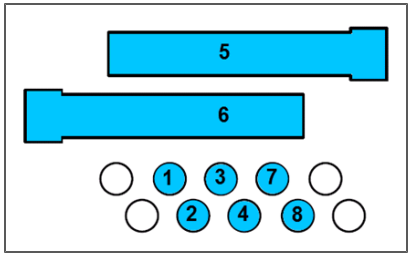
96 Samples, Box 2 of 2

You will receive either a Ribo-Zero Human/Mouse/Rat box or Ribo-Zero Gold box 2, depending on the kit ordered.

Store as specified

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

Figure 17 TruSeq Stranded Total RNA HT Sample Prep Kit, 96 Samples (Box 2 of 2)



Ribo-Zero H/M/R Box

Table 10 Ribo-Zero Human/Mouse/Rat, part # 15032626

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

Ribo-Zero Gold Box

Table 11 Ribo-Zero Gold, part # 15032627

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

Consumables and Equipment

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

Table 12 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 - for alliquotting reagents)	Thomson Instrument Company, part # 951652

Consumable	Supplier
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
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	BioRad, part # MSB-1001
Nuclease-free ultra pure water	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
RNase/DNase-free 8-well PCR strip tubes and caps	General lab supplier
RNase/DNase zapper (to decontaminate surfaces)	General lab supplier
SuperScript II Reverse Transcriptase	Invitrogen, part # 18064-014
Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20	General lab supplier
Tween 20	Sigma, part # P7949

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

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

Table 14 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 15 User-Supplied Equipment

Equipment	Supplier
96-well thermal cycler (with heated lid)	General lab supplier
Magnetic stand-96	Life Technologies, part # AM10027
Microplate centrifuge	General lab supplier
Vortexer	General lab supplier

Table 16 User-Supplied Equipment - Additional Items for HS Processing

Consumable	Supplier
High Speed Micro Plate Shaker	VWR, catalog # 13500-890 (110V/120V) catalog # 14216-214 (230V)
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
Tru Temp Microheating System Note: Two systems are recommended to support successive heating procedures.	Illumina, catalog # SC-60-503 (115V) catalog # SC-60-504 (220V)

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 the indexed adapter sequences. The set (A or B) containing the adapter is also specified.

NOTE

- The index numbering is not contiguous. Index 17, 24, and 26 are skipped.
- The base in parentheses () indicates the base for the seventh cycle and is not considered as part of the index sequence. The index should be recorded in the sample sheet as only six bases. For indexes 13 and above, the seventh base (in parentheses) might not be A, and this will be seen in the seventh cycle of the index read.

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

Adapter	Sequence	Set		Adapter	Sequence	Set
AR001	ATCACG(A)	B		AR013	AGTCAA(C)	A
AR002	CGATGT(A)	A		AR014	AGTTCC(G)	A
AR003	TTAGGC(A)	B		AR015	ATGTCA(G)	A
AR004	TGACCA(A)	A		AR016	CCGTCC(C)	A
AR005	ACAGTG(A)	A		AR018	GTCCGC(A)	A
AR006	GCCAAT(A)	A		AR019	GTGAAA(C)	A
AR007	CAGATC(A)	A		AR020	GTGGCC(T)	B

Adapter	Sequence	Set		Adapter	Sequence	Set
AR008	ACTTGA(A)	B		AR021	GTTTCG(G)	B
AR009	GATCAG(A)	B		AR022	CGTACG(T)	B
AR010	TAGCTT(A)	B		AR023	GAGTGG(A)	B
AR011	GGCTAC(A)	B		AR025	ACTGAT(A)	B
AR012	CTTGTA(A)	A		AR027	ATTCCT(T)	B

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 the 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 Sequences

Indexed Adapter 1	Sequence	Indexed Adapter 2	Sequence
D701	ATTACTCG	D501	TATAGCCT
D702	TCCGGAGA	D502	ATAGAGGC
D703	CGCTCATT	D503	CCTATCCT
D704	GAGATTCC	D504	GGCTCTGA
D705	ATTCAGAA	D505	AGGCGAAG
D706	GAATTCGT	D506	TAATCTTA

Indexed Adapter 1	Sequence	Indexed Adapter 2	Sequence
D707	CTGAAGCT	D507	CAGGACGT
D708	TAATGCGC	D508	GTACTGAC
D709	CGGCTATG		
D710	TCCGCGAA		
D711	TCTCGCGC		
D712	AGCGATAG		

Adapter Options

Illumina provides two methods for indexing samples to perform pooled sequencing, using either Adapter Index tubes or a RAP.

Adapter Tubes

The TruSeq Stranded Total RNA LT Sample Prep Kit contains Adapter Index tubes that can be used to perform pooled sequencing.

- Each tube contains a unique single 6 base index adapter on the P7 strand and contains enough reagent for eight reactions.
- Samples prepared with these adapters can be sequenced on any Illumina sequencing platform using the 7 cycle Single Index Recipe.

For more information on pooling guidelines when using adapter index tubes, see *Adapter Tube Pooling Guidelines* on page 51.

For more information on sequencing samples prepared using the TruSeq Stranded Total RNA LT Sample Prep Kit, see your sequencing platform user guide.

Adapter Plate

The TruSeq Stranded Total RNA HT Sample Prep Kit contains a RAP, which is a 96-well plate containing 96 uniquely indexed adapter combinations designed for manual or automated preparation of 96 uniquely indexed samples.

- Each well of the plate is single-use and the plate can undergo up to 4 freeze-thaw cycles.
- The adapters provided in this plate are dual-indexed, meaning that each adapter contains two indices. These are referred to as Index 1(i7), an 8 base Index on the P7 strand, and Index 2(i5), an 8 base Index on the P5 strand.
- There are 12 Index 1 sequences (D701-D712) arrayed across the columns and 8 Index 2 sequences (D501-D508) arrayed down the rows, to generate 96 uniquely dual-indexed adapter combinations in the plate.
- If compatible, samples prepared with these adapters can be sequenced on an Illumina sequencing platform using the dual-indexed recipes for dual indexing or the 8 cycle single-indexed recipe for single indexing.

For more information on pooling guidelines when using the RAP, see *Adapter Plate Pooling Guidelines* on page 52.

For more information on sequencing samples prepared using the TruSeq Stranded Total RNA HT Sample Prep Kit, see your sequencing platform user guide.

Table 19 Dual-Indexed Sequencing Platform Compatibility

Platform	Compatibility
MiSeq®	Full compatibility
HiSeq®	• Requires TruSeq Dual Index Sequencing Primer Box, Single Read for dual-indexed sequencing on a single-read flow cell.[a] • Requires HCS 1.5/RTA 1.13 or later • Process with OLB 1.9.3 or later if offline base call is needed • Process with CASAVA 1.8.2 or later
Genome Analyzer™	• Requires TruSeq Dual Index Sequencing Primer Box, Single Read for dual-indexed sequencing on a single-read flow cell.[a] • Requires SCS 2.10/RTA 1.13 or later • Process with OLB 1.9.4 or later if offline base call is needed • Process with CASAVA 1.8.2 or later

a. Not required for sequencing on paired-end flow cells.

Pooling Preparation with Adapter Plate

The TruSeq Stranded Total RNA HT Sample Prep Kit contains a RAP and enables preparation of up to 96 libraries with unique dual indexes.

Figure 18 RAP Dual-Indexed Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	D701-D501	D702-D501	D703-D501	D704-D501	D705-D501	D706-D501	D707-D501	D708-D501	D709-D501	D710-D501	D711-D501	D712-D501
B	D701-D502	D702-D502	D703-D502	D704-D502	D705-D502	D706-D502	D707-D502	D708-D502	D709-D502	D710-D502	D711-D502	D712-D502
C	D701-D503	D702-D503	D703-D503	D704-D503	D705-D503	D706-D503	D707-D503	D708-D503	D709-D503	D710-D503	D711-D503	D712-D503
D	D701-D504	D702-D504	D703-D504	D704-D504	D705-D504	D706-D504	D707-D504	D708-D504	D709-D504	D710-D504	D711-D504	D712-D504
E	D701-D505	D702-D505	D703-D505	D704-D505	D705-D505	D706-D505	D707-D505	D708-D505	D709-D505	D710-D505	D711-D505	D712-D505
F	D701-D506	D702-D506	D703-D506	D704-D506	D705-D506	D706-D506	D707-D506	D708-D506	D709-D506	D710-D506	D711-D506	D712-D506
G	D701-D507	D702-D507	D703-D507	D704-D507	D705-D507	D706-D507	D707-D507	D708-D507	D709-D507	D710-D507	D711-D507	D712-D507
H	D701-D508	D702-D508	D703-D508	D704-D508	D705-D508	D706-D508	D707-D508	D708-D508	D709-D508	D710-D508	D711-D508	D712-D508

When less than the full set of 96 libraries are pooled and sequenced, it is extremely important that libraries with compatible index combinations are used in the indexed pool. Illumina strongly recommends the following planning steps before beginning library preparation:

1 Determine the number of libraries that will be pooled for sequencing.

2 Ensure that the pool contains the required index combinations, as described in *Adapter Plate Pooling Guidelines* on page 52. Select the RNA index adapters based on the same guidelines.

3 Use the Illumina Experiment Manager to create a sample sheet which will be used during the sequencing run. This step also identifies any incorrect index combinations, allowing re-design before library preparation starts. For more information, see *Tracking Tools* on page 29.

4 Use the Lab Tracking Form or sample plate generator from the Illumina Experiment Manager to specify the layout of all sample plates in 96-well plate format for compatibility with the 96-well RAP. Arrange samples that will be pooled together in the same orientation as the indices in the RAP. For more information, see *Tracking Tools* on page 29.

Handling Adapter Plate

The RAP is designed for use in the TruSeq Stranded Total RNA Sample Prep high sample protocol.

- The RAP is single-use for each well.

- Illumina recommends that the RAP does not undergo more than 4 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.

Prepare Adapter Plate

Prepare the RAP for use as follows:

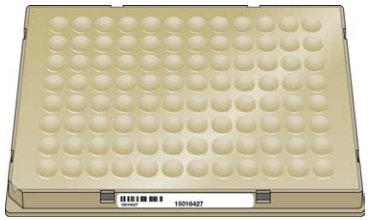
1. Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to ensure that they all are completely thawed.
2. Remove the adapter plate tape seal.
3. Centrifuge the plate at 280 xg for 1 minute to collect all of the adapter to the bottom of the well.
4. Remove the plastic cover and save the cover if you are not processing the entire plate at once.
5. Apply the RAP barcode label to the RAP.
   If using only part of the RAP, it may be useful to use a lab pen to mark on the foil seal the adapter wells being used. When doing so, be careful not to pierce the foil seal.

Pierce Adapter Plate Seal

Pierce the RAP foil seal as follows:

1. 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 located on the lower left.

   Figure 19 Correct RAP Orientation

2. Do one of the following:

  - If using the entire plate at once, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously by gently but firmly pressing 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 desired columns that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each column of adapters that will be used for ligation.
- Once the foil seal has been pierced for a well, Illumina does not recommend reusing the dual-indexed adapter from that well in future sample preparations.

Pipette Adapter Plate

Pipette the adapters from the RAP into the ligation reaction as follows, while keeping the plate in the same orientation:

1. Using an 8-tip multichannel pipette, transfer the thawed adapter from the RAP well to each well of the sample plate.
2. Change pipette tips between wells of the RAP. This is critical to avoid cross-contamination between wells.
3. Aspirate each dual-indexed adapter by column.
4. Discard the tips after pipetting into the ligation reaction.

Adapter Plate Storage

If not all adapter wells are used in a single experiment (< 96 samples), the plate can be stored for future use of unused wells as follows:

1. Wipe the foil seal covering unused wells with a sterile 70% Ethanol wipe.
2. Allow the foil seal to dry.
3. Put the plastic cover that came with the RAP back on the plate.
4. Store at -15° to -25°C.

NOTE

Do not reseal the plate with a disposable seal. This will rip the original foil seal when the disposable seal is removed for future uses.

Pooling Guidelines

Illumina uses a green laser to sequence G/T and a red laser to sequence A/C. At each cycle at least one of two nucleotides for each color channel needs to be read to ensure proper image registration. It is important to maintain color balance for each base of the index read being sequenced, otherwise index read sequencing could fail due to registration failure. Follow these low plex pooling guidelines, depending on the TruSeq Stranded Total RNA Sample Prep kit you are using.

Adapter Tube Pooling Guidelines

When using the index adapter tubes from the TruSeq Stranded Total RNA LT Sample Prep Kit, follow these pooling guidelines for single-indexed sequencing. The TruSeq Stranded Total RNA LT Sample Prep Kit Set A and B, each contain 12 unique index adapter tubes. When designing low-plexity index pools for single-indexed sequencing, always use at least two unique and compatible barcodes for each index sequenced. The following table describes possible pooling strategies for 2–4 samples generated with the adapter index tubes in each set.

- For 5–11plex pools, use 4-plex options with any other available adapters
- Not all color-balanced pools are listed. Check the color balance of such user-designed pools using the Illumina Experiment Manager's sample sheet generator.

Table 20 Single-Indexed Pooling Strategies for 2–4 Samples

Plexity	Option	Set A Only	Set B Only
2	1	AR006 and AR012	Not recommended
	2	AR005 and AR019	
3	1	AR002 and AR007 and AR019	AR001 and AR010 and AR020
	2	AR005 and AR006 and AR015	AR003 and AR009 and AR025
	3	2-plex options with any other adapter	AR008 and AR011 and AR022
4	1	AR005 and AR006 and AR012 and AR019	AR001 and AR008 and AR010 and AR011
	2	AR002 and AR004 and AR007 and AR016	AR003 and AR009 and AR022 and AR027
	3	3-plex options with any other adapter	3-plex options with any other adapter

For more information on the Single-Indexed Sequencing workflow, see the Illumina HiSeq, HiScan®, and Genome Analyzer user guides.

Adapter Plate Pooling Guidelines

When using the the RAP from the TruSeq Stranded Total RNA HT Sample Prep Kit, follow these pooling guidelines. In addition, please review *Handling Adapter Plate* on page 48 and *Pooling Preparation with Adapter Plate* on page 47.

Single-Indexed Sequencing

Follow the single-indexed sequencing workflow when pooling 12 or fewer samples. When designing low plexity index pools, always use at least two unique and compatible barcodes for each index sequenced. The following figures illustrate possible pooling strategies for 2–12 samples generated with the RAP.

- Color balanced pools are shaded light gray with green wells.
- For 5-plex pools, dark gray wells are not used for pooled sequencing. They are available for individual sequencing.
- For 7–11plex pools, combine any of the 2–6plex pools.
- Not all color-balanced pools are illustrated. Check the color balance of such user-designed pools using the Illumina Experiment Manager's sample sheet generator.

For more information on the single-indexed sequencing workflow, see the Illumina HiSeq, HiScan, and Genome Analyzer user guides.

Figure 20 Single-Indexed–2-plex

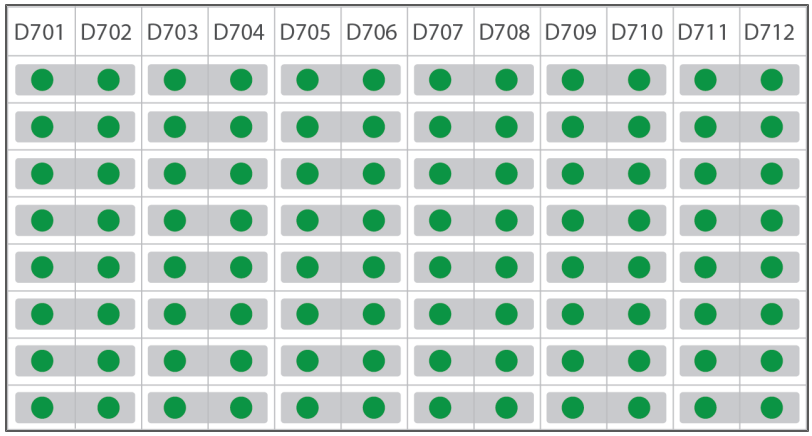


Figure 21 Single-Indexed–3-plex

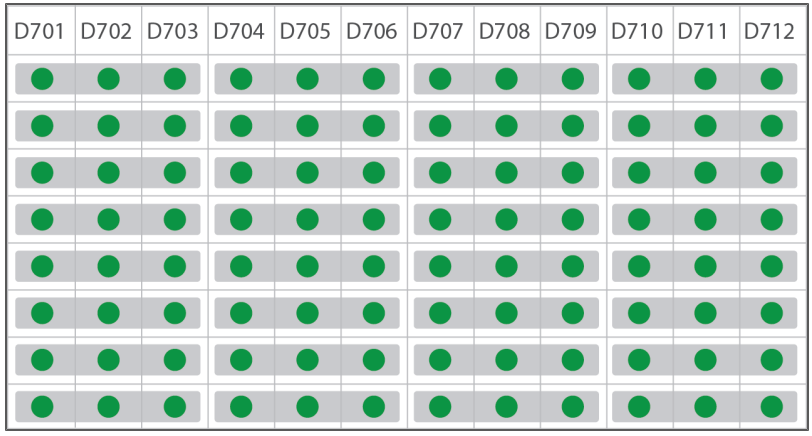


Figure 22 Single-Indexed–4-plex

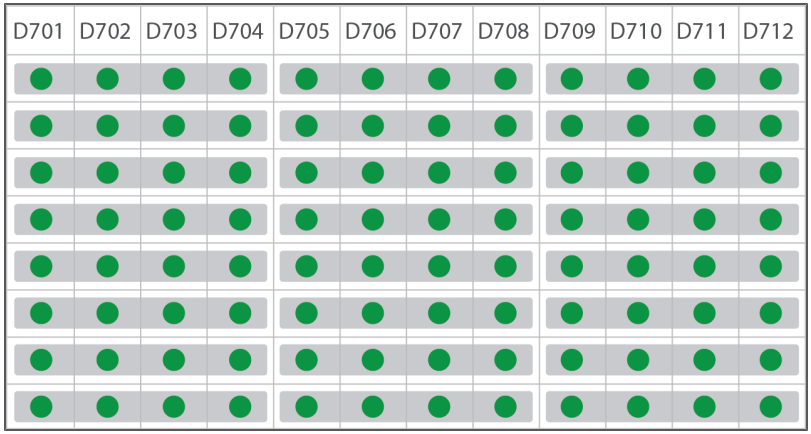


Figure 23 Single-Indexed–5-plex

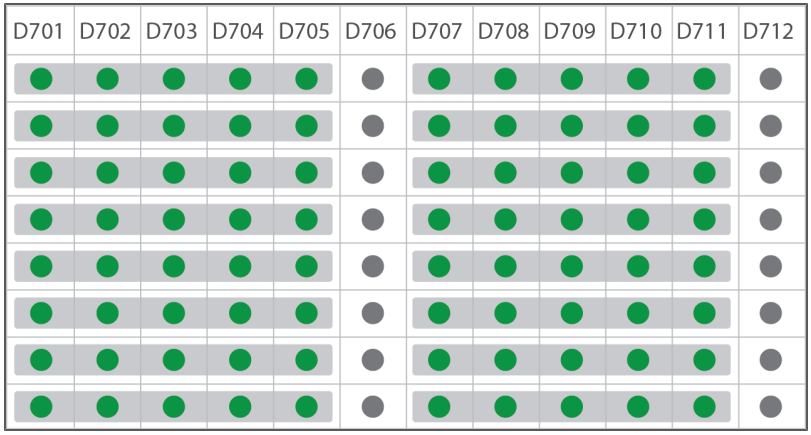


Figure 24 Single-Indexed–6-plex

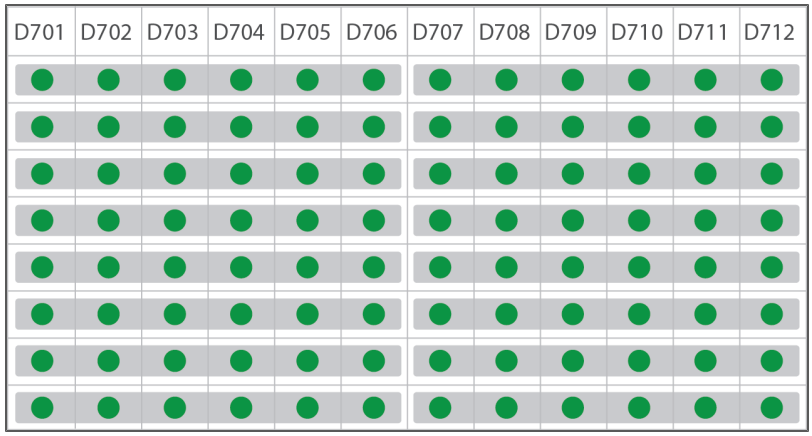
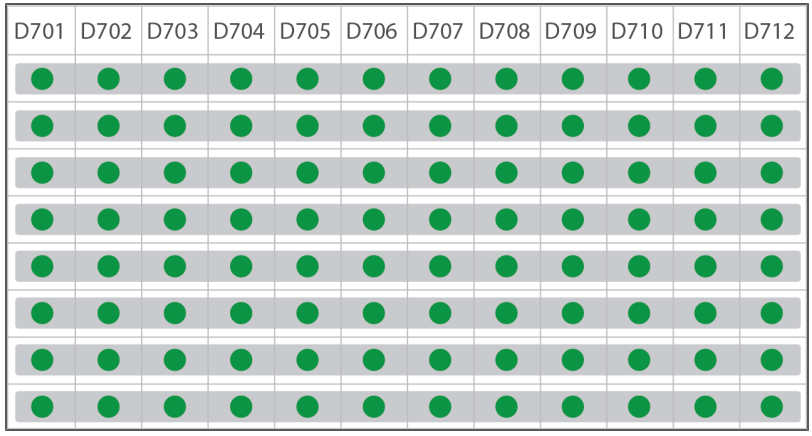


Figure 25 Single-Indexed–12-plex



Dual-Indexed Sequencing

Follow the dual-indexed sequencing workflow when pooling more than 12 samples. When designing the low-plexity index pools, always use at least two unique and compatible barcodes for each index sequenced. The following figures illustrate possible pooling strategies for 2–16 samples generated with the RAP.

- Color balanced pools are shaded light gray with green wells. The 2-plex pools are diagonal and shaded in light or dark gray with green wells.
- Odd numbered pools display dark gray wells that are not used for pooled sequencing. They are available for individual sequencing.
- Not all color-balanced pools are illustrated. Check the color balance of such user-designed pools using the Illumina Experiment Manager's sample sheet generator.

For more information on the dual-indexed sequencing workflow, see the Illumina HiSeq, HiScan, Genome Analyzer, and MiSeq user guides.

Figure 26 Dual-Indexed–2-plex

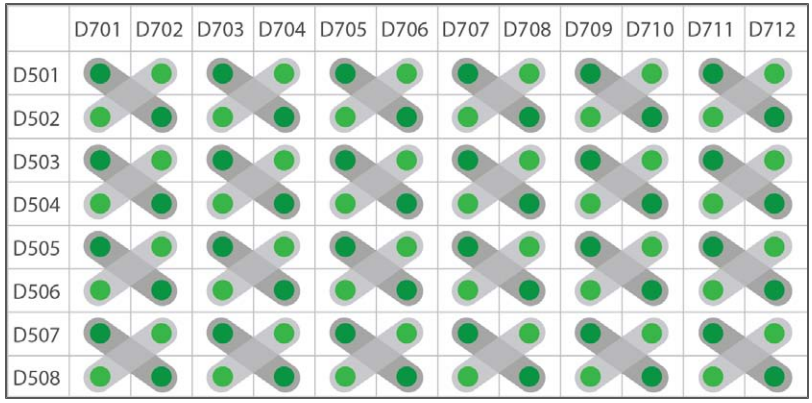


Figure 27 Dual-Indexed–3-plex

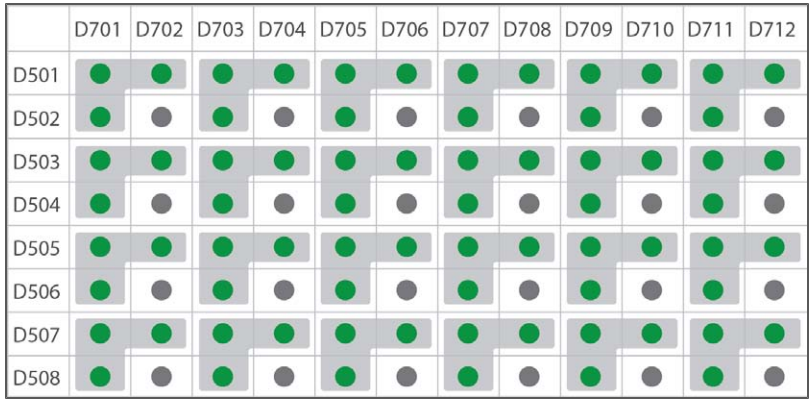


Figure 28 Dual-Indexed–4-plex

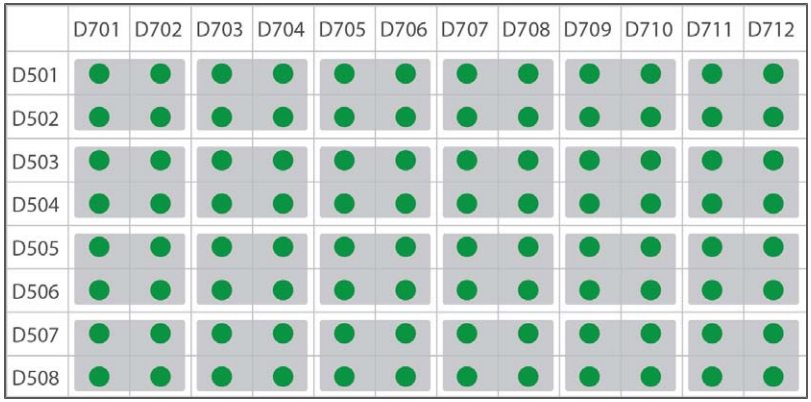


Figure 29 Dual-Indexed–5-plex

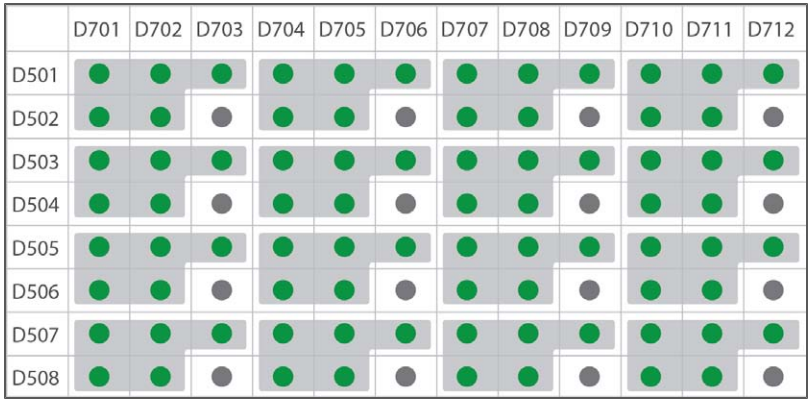


Figure 30 Dual-Indexed–6-plex

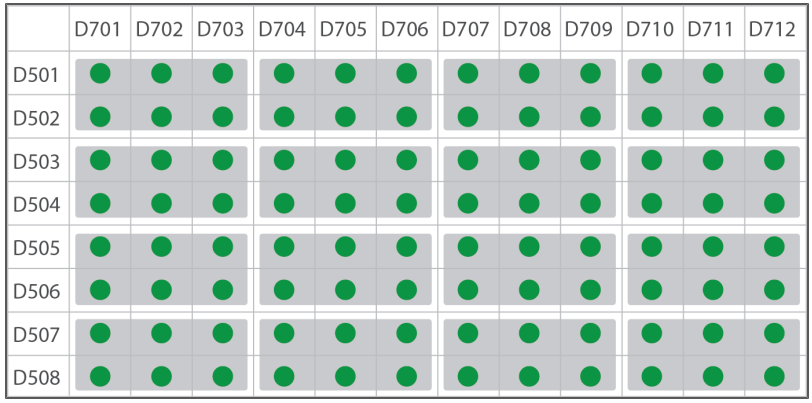


Figure 31 Dual-Indexed–7-plex

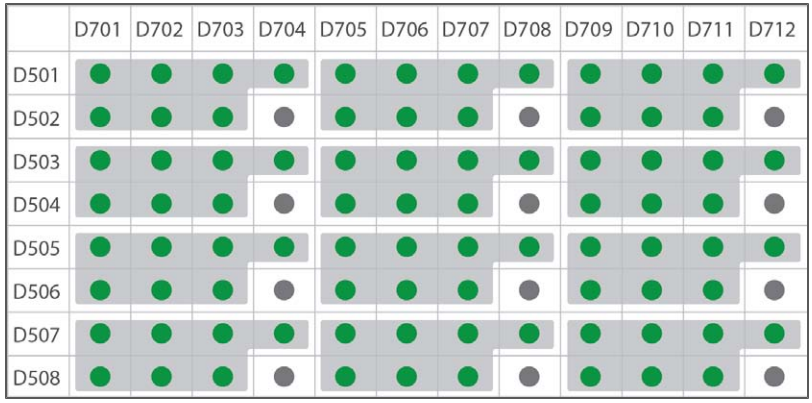


Figure 32 Dual-Indexed–8-plex, Option 1

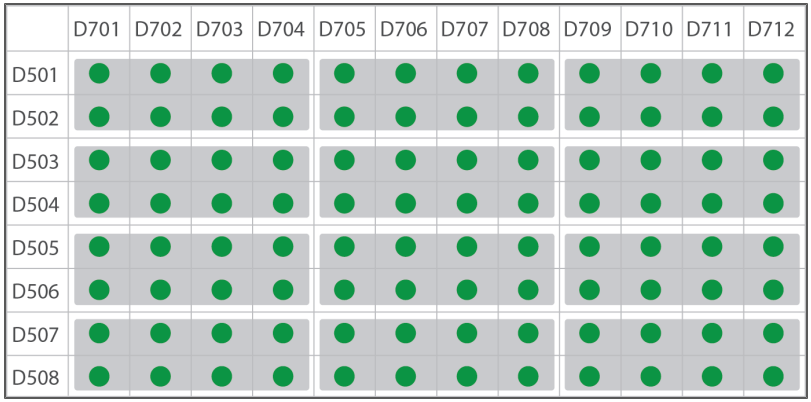


Figure 33 Dual-Indexed–8-plex, Option 2

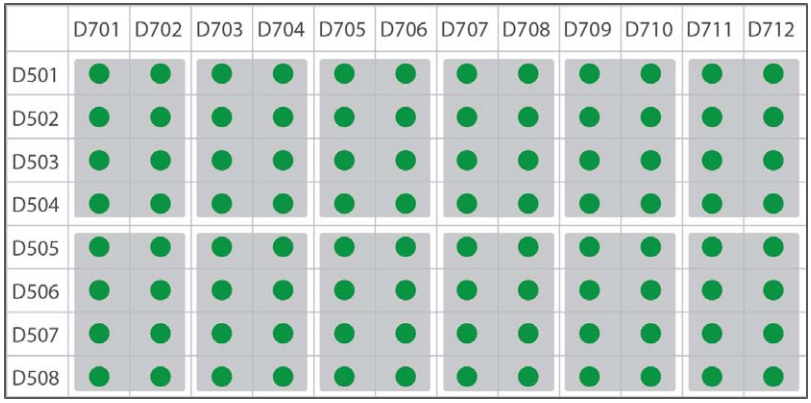


Figure 34 Dual-Indexed–12-plex

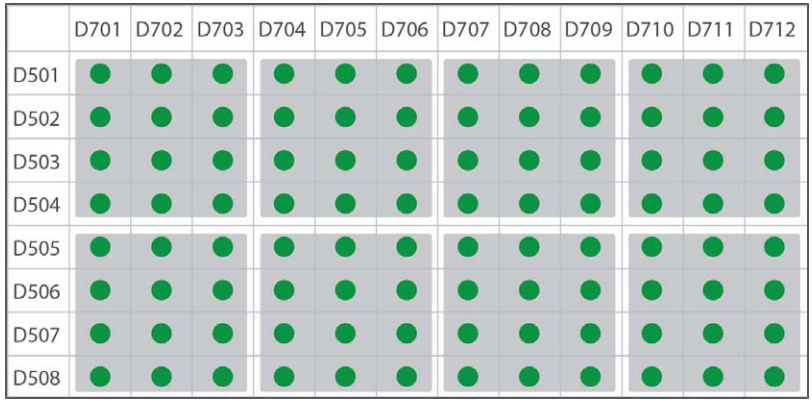
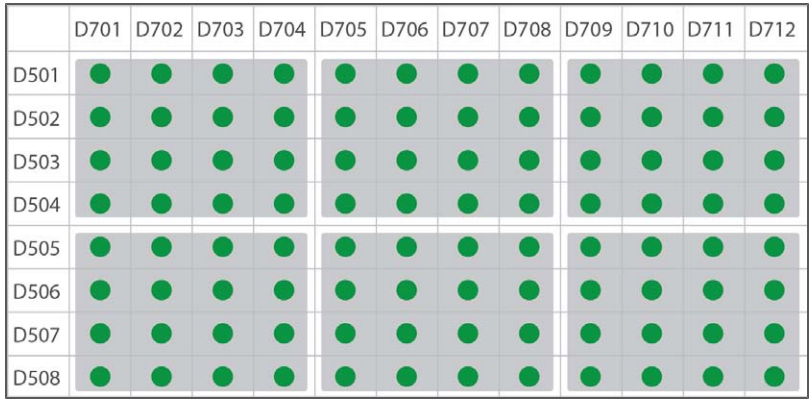


Figure 35 Dual-Indexed–16-plex



Low Sample (LS) Protocol

Chapter 3

Introduction 62
Sample Prep Workflow 64
Ribo-Zero Deplete and Fragment RNA 65
Synthesize First Strand cDNA 73
Synthesize Second Strand cDNA 76
Adenylate 3' Ends 80
Ligate Adapters 82
Enrich DNA Fragments 89
Validate Library 93
Normalize and Pool Libraries 95

Introduction

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

Table 21 Kit and Sample Number Recommendations

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

Table 22 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

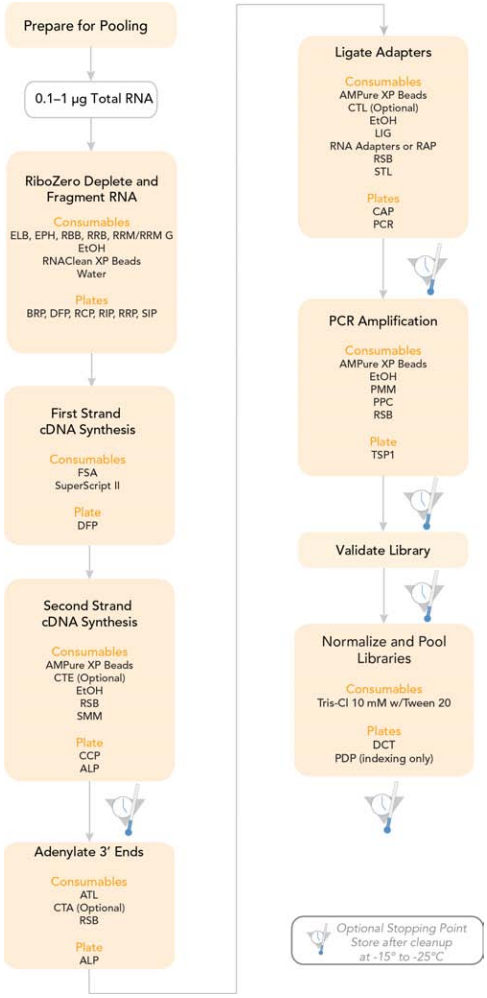
- Review *Best Practices* on page 12 before proceeding.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample preparation. For more information, see *Tracking Tools* on page 29.

- If you are pooling using adapter index tubes, record information about your samples before beginning library preparation for later use in data analysis. For more information, see *Tracking Tools* on page 29. Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
- If you are pooling with the RAP, please review the planning steps in *Pooling Preparation with Adapter Plate* on page 47 before beginning library preparation.

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 36 TruSeq Stranded Total RNA Sample Preparation LS Workflow

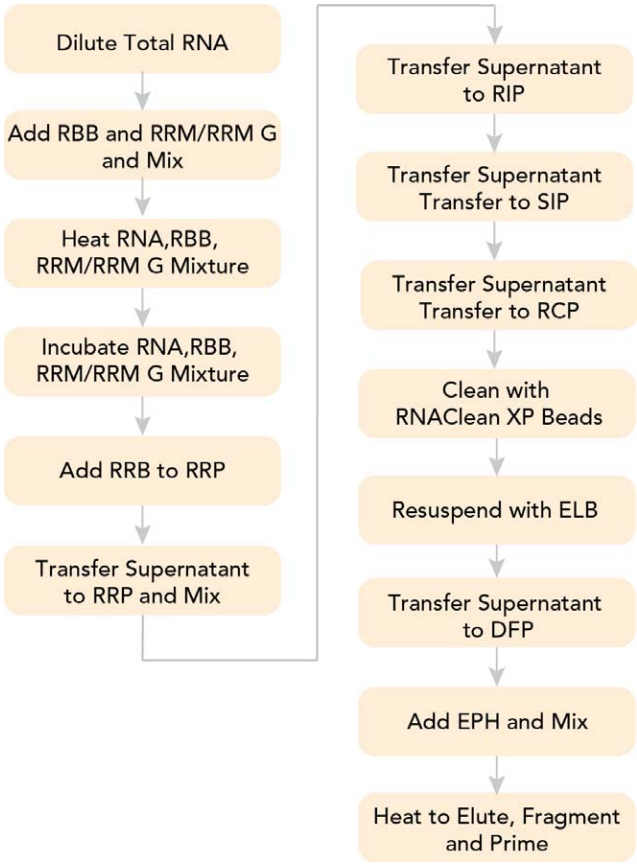


Ribo-Zero Deplete and Fragment RNA

This process depletes ribosomal RNA from total RNA. After the ribosomal RNA is depleted, the remaining RNA is purified, fragmented and primed for cDNA synthesis.

Reference the following diagram while performing the purification procedures:

Figure 37 TruSeq Stranded Total RNA Sample Prep Purification Workflow



It is important to follow this procedure exactly to be sure of reproducibility.

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
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.

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° to -25°C	Illumina
Elution Buffer (ELB)	1 tube per 48 reactions	2° to 8°C	Illumina
One of the following, depending on the kit you are using: • Ribo-Zero rRNA Removal Mix (RRM) (Ribo-Zero Human/Mouse/Rat kit contents) • Ribo-Zero rRNA Removal Mix - Gold (RRM G) (Ribo-Zero Gold kit contents)	1 tube per 48 reactions	-15° to -25°C	Illumina

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	-15° to -25°C	Illumina
rRNA Binding Buffer (RBB)	1 tube per 48 reactions	2° to 8°C	Illumina
rRNA Removal Beads (RRB)	1 tube per 48 reactions	2° to 8°C	Illumina
BRP (Bind rRNA Plate) barcode label	1 label per plate	15° to 30°C	Illumina
DFP (Depleted RNA Fragmentation Plate) barcode label	1 label per plate	15° to 30°C	Illumina
RCP (RNA Clean Up Plate) barcode label	1 label per plate	15° to 30°C	Illumina
RIP (Removal Intermediate Plate) barcode label	1 label per plate	15° to 30°C	Illumina
RRP (rRNA Removal Plate) barcode label	1 label per plate	15° to 30°C	Illumina
SIP (Second Removal Intermediate Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well 0.3 ml PCR plates	6	15° to 30°C	User
Freshly Prepared 70% Ethanol (EtOH)	200 µl per sample	15° to 30°C	User
Microseal 'B' Adhesive Seals	2	15° to 30°C	User
RNAClean XP Beads	99 µl per sample	2° to 8°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	6	15° to 30°C	User

Item	Quantity	Storage	Supplied By
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	6	15° to 30°C	User
Ultra Pure Water	Enough to dilute each total RNA sample to a final volume of 10 µl	15° to 30°C	User

Preparation

- Remove the following from -15° 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:
    - Ribo-Zero rRNA Removal Mix
    - Ribo-Zero rRNA Removal Mix - Gold
  - Resuspension Buffer

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

- Remove the following from 2° to 8°C storage and let stand to bring to room temperature:
  - Elution Buffer
  - rRNA Binding Buffer
  - rRNA Removal Beads
- Remove the RNAClean XP beads from 2° 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
  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° 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 a RIP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a RCP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a RRP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a SIP 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. Do one of the following, depending on the kit you are using:
   - Add 5 µl of Ribo-Zero rRNA Removal Mix to each well of the BRP plate.
   - Add 5 µl of Ribo-Zero rRNA Removal Mix - Gold to each well of the BRP plate.
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 rRNA Binding Buffer to 2° to 8°C storage.
7. Do one of the following, depending on the kit you are using:
   - Return the Ribo-Zero rRNA Removal Mix to -15° to -25°C storage.
   - Return the Ribo-Zero rRNA Removal Mix - Gold to -15° to -25°C storage.

Incubate 1 BRP

1. Place the sealed BRP plate on the pre-programmed thermal cycler. Close the lid and select **RNA Denaturation** to denature the RNA.
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 completely resuspend the beads.

2 Add 35 µl 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 to not 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 and transfer the entire contents (20 µl) from each well of the BRP plate to the corresponding well of the RRP plate containing rRNA Removal Beads.

4 Adjust the pipette to 45 µl, 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 ribosomal RNA 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 since the foam is not transferred out of the plate efficiently.

5 Incubate the RRP plate at room temperature for 1 minute.

6 Place the RRP plate on the magnetic stand at room temperature for 1 minute.

7 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 RIP barcode.

8 Place the RIP plate on the magnetic stand at room temperature for 1 minute.

9 Transfer all of the supernatant from each well of the RIP plate to the corresponding well of the new 96-well 0.3 ml PCR plate labeled with the SIP barcode.

10 Place the SIP plate on the magnetic stand at room temperature for 1 minute.

11 Transfer all of the supernatant from each well of the SIP plate to the corresponding well of the new 96-well 0.3 ml PCR plate labeled with the RCP barcode.

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.

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

Clean Up RCP

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 14 when working with RNAClean XP beads.

1 Vortex the RNAClean XP beads until they are well dispersed, then add 99 µl 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 µl 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 remaining on the magnetic stand, add 200 µl of freshly prepared 70% EtOH to each well without disturbing the beads.

6 Incubate the RCP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

7 Let the plate stand at room temperature for 15 minutes to dry and then remove the RCP plate from the magnetic stand.

8 Centrifuge the thawed, room temperature Elution Buffer to 600 xg for 5 seconds.

9 Add 11 µl 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° to 8°C storage.

13 Transfer 8.5 µl of the supernatant from the RCP plate to the new 96-well 0.3 ml PCR plate labeled with the DFP barcode.

14 Add 8.5 µl 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° to -25°C storage and the RNAClean XP Beads tube to 2° 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.

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 73.

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.

Consumables

Item	Quantity	Storage	Supplied By
First Strand Synthesis Act D Mix (FSA)	1 tube per 48 reactions	-15° to -25°C	Illumina
96-well 0.3 ml PCR plate	1	15° to 30°C	User
Microseal 'B' Adhesive Seal	1	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	1	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	1	15° to 30°C	User
SuperScript II Reverse Transcriptase	1 tube	-15° to -25°C	User

WARNING
First Strand Synthesis Mix Act D (FSA) 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. Please 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 http://www.illumina.com/msds.

Preparation

- Remove one tube of First Strand Synthesis Act D Mix from -15° 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° 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 xg 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° 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**.
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 76.

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. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix. At the end of this process, you will have blunt-ended cDNA.

Consumables

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

Preparation

- Remove the following from -15° 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° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 14.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the thermal cycler to 16°C.
- Choose the thermal cycler pre-heat lid option and set to 30°C
- Apply a 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 xg 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 xg 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° 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, remove the adhesive seal, and let stand to bring the plate to room temperature.

Clean Up DFP

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 14 when working with AMPure XP Beads.

1 Vortex the AMPure XP beads until they are well dispersed, then 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.

2 Incubate the DFP plate at room temperature for 15 minutes.

3 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.

4 Remove and discard 135 µl of the 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 (5–7).

5 With the DFP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

6 Incubate the DFP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

7 Repeat steps 5 and 6 once for a total of two 80% EtOH washes.

8 Let the plate stand at room temperature for 15 minutes to dry and then remove the DFP plate from the magnetic stand.

9 Centrifuge the thawed, room temperature Resuspension Buffer to 600 xg for 5 seconds.

10 Add 17.5 µl Resuspension Buffer to each well of the DFP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

11 Incubate the DFP plate at room temperature for 2 minutes.

12 Place the DFP plate on the magnetic stand at room temperature for 5 minutes.

13 Transfer 15 µl of the supernatant (ds cDNA) from the DFP plate to the new 96-well 0.3 ml PCR plate labeled with the ALP barcode.

SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3′ Ends* on page 80 immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven 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° to -25°C	Illumina
A-Tailing Mix (ATL)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Microseal 'B' Adhesive Seal	1	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	3	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	3	15° to 30°C	User

Preparation

- Remove the following from -15° 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° to 8°C storage and bring it to room temperature.
- Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up DFP* on page 78 and let stand to thaw at room temperature.
  - Centrifuge the thawed ALP plate to 280 xg 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 xg 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 and select **ATAIL70**.
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 82.

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° to -25°C	Illumina

Choose from the following depending on the kit you are using:
• TruSeq Stranded Total RNA LT Sample Prep Kit contents:
  • RNA Adapter Indices (AR001–AR016, AR018–AR023, AR025, AR027)
• TruSeq Stranded Total RNA HT Sample Prep Kit contents:
  • RAP (RNA Adapter Plate)
	1 tube per column of 8 reactions, of each indices being used
	or
	1 RAP
		-15° to -25°C	Illumina

Ligation Mix (LIG)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Stop Ligation Buffer (STL)	1 tube per 48 reactions	-15° to -25°C	Illumina
CAP (Clean Up ALP Plate) barcode label	1 label per plate	15° to 30°C	Illumina
PCR (Polymerase Chain Reaction) barcode label	1 label per plate	15° to 30°C	Illumina

Item	Quantity	Storage	Supplied By
RAP (RNA Adapter Plate) barcode label (if using the HT kit)	1 label per plate	15° to 30°C	Illumina
96-well 0.3 ml PCR plates	2	15° to 30°C	User
AMPure XP beads	92 µl per sample	2° to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	800 µl per sample	15° to 30°C	User
Microseal 'B' Adhesive Seals	2	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	4–28	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	4–28	15° to 30°C	User

Preparation

- Remove the following from -15° 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.
    - If using the RAP, review *Handling Adapter Plate* on page 48.
  - Stop Ligation Buffer

NOTE
Do not remove the Ligation Mix tube from -15° 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° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 14.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- 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.

NOTE

- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate 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

Illumina recommends that the RAP does not undergo more than 4 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.

Add LIG

1 Do one of the following:

- If using RNA Adapter tubes, centrifuge the appropriate/desired thawed tubes to 600 xg for 5 seconds.
- If using a RAP:
  - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to ensure that they all are completely thawed.
  - Remove the adapter plate tape seal.
  - Centrifuge the plate at 280 xg for 1 minute to collect all of the adapter to the bottom of the well.
  - Remove the plastic cover and save the cover if you are not processing the entire plate at once.

- If this 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 xg for 5 seconds.

3 Immediately before use, remove the Ligation Mix tube from -15° to -25°C storage.

4 Remove the adhesive seal from the ALP plate.

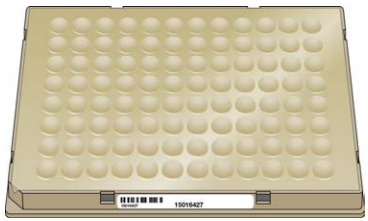
5 Do one of the following:
- If using the in-line control reagent:
  - Dilute the Ligation Control to 1/100 in Resuspension Buffer (For example, 1 µl Ligation Control + 99 µl Resuspension Buffer) before use. Discard the diluted Ligation Control after use.
  - Add 2.5 µl of diluted Ligation Control to 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.

6 Add 2.5 µl of Ligation Mix to each well of the ALP plate.

7 Return the Ligation Mix tube back to -15° to -25°C storage immediately after use.

8 Do one of the following:
- If using RNA Adapter tubes, add 2.5 µl of the appropriate/desired 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 located on the lower left.

Figure 38 Correct RAP Orientation



  - Do one of the following to pierce the foil seal:

- If using the entire plate at once, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously by gently but firmly pressing 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 desired columns that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each column of adapters that will be used for ligation.

– Using an 8-tip multichannel pipette, transfer 2.5 µl of the appropriate/desired 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 xg 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 µl 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

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 14 when working with AMPure XP Beads.

1 Vortex the AMPure XP Beads until they are well dispersed, then add 42 µl 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.

2 Incubate the ALP plate at room temperature for 15 minutes.

3 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

4 Remove and discard 79.5 µl of the supernatant from each well of the ALP plate.

NOTE
Leave the ALP plate on the magnetic stand while performing the following 80% EtOH wash steps (5–7).

5 With the ALP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

6 Incubate the ALP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

7 Repeat steps 5 and 6 once for a total of two 80% EtOH washes.

8 While keeping the ALP 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.

9 Resuspend the dried pellet in each well with 52.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.

10 Incubate the ALP plate at room temperature for 2 minutes.

11 Place the ALP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

12 Transfer 50 µl of the clear 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.

13 Vortex the AMPure XP Beads until they are well dispersed, then add 50 µl of mixed AMPure XP Beads to each well of the CAP plate for a second clean up. Gently pipette the entire volume up and down 10 times to mix thoroughly.

14 Incubate the CAP plate at room temperature for 15 minutes.

15 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

16 Remove and discard 95 µl of the supernatant from each well of the CAP plate.

NOTE
Leave the CAP plate on the magnetic stand while performing the following 80% EtOH wash steps (17–19)

17 With the CAP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

18 Incubate the CAP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

19 Repeat steps 17 and 18 once for a total of two 80% EtOH washes.

20 While keeping 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.

21 Resuspend the dried pellet in each well with 22.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.

22 Incubate the CAP plate at room temperature for 2 minutes.

23 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

24 Transfer 20 µl of the clear 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.

SAFE STOPPING POINT
If you do not plan to proceed to *Enrich DNA Fragments* on page 89 immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -15° 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. The number of PCR cycles should be minimized 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, as fragments without any adapters cannot hybridize to surface-bound primers in the flow cell, and 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° to -25°C	Illumina
PCR Primer Cocktail (PPC)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
TSP1 (Target Sample Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well 0.3 ml PCR plate	1	15° to 30°C	User
AMPure XP beads	50 µl per sample	2° to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	15° to 30°C	User
Microseal 'B' Adhesive Seals	2	15° to 30°C	User

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

Preparation

- Remove the PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature.
- Centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- Remove the Resuspension Buffer from 2° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 14.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the PCR plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 86 and let stand to thaw at room temperature.
  - Centrifuge the thawed PCR plate to 280 xg 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 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. 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 and select **PCR** to amplify the plate.

Clean Up PCR

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 14 when working with AMPure XP Beads.

1. Remove the adhesive seal from the PCR plate.
2. Vortex the AMPure XP Beads until they are well dispersed, then do one of the following:
   - If using the RNA Adapter tubes, add 50 µl of the mixed AMPure XP Beads to each well of the PCR plate containing 50 µl 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 µl of the mixed AMPure XP Beads to each well of the PCR plate containing 50 µl of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.
3. Incubate the PCR plate at room temperature for 15 minutes.
4. Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.
5. Remove and discard 95 µl of the supernatant from each well of the PCR plate.

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

6 With the PCR plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

7 Incubate the PCR plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

8 Repeat steps 6 and 7 once for a total of two 80% EtOH washes.

9 While keeping 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.

10 Resuspend the dried pellet in each well with 32.5 µl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.

11 Incubate the PCR plate at room temperature for 2 minutes.

12 Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

13 Transfer 30 µl of the clear 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.

SAFE STOPPING POINT

If you do not plan to proceed to *Validate Library* on page 93 immediately, the protocol can be safely stopped here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven 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

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

Quality Control

1. Load 1 µl 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 39 Example of TruSeq Stranded Total RNA Sample Prep Library Size Distribution

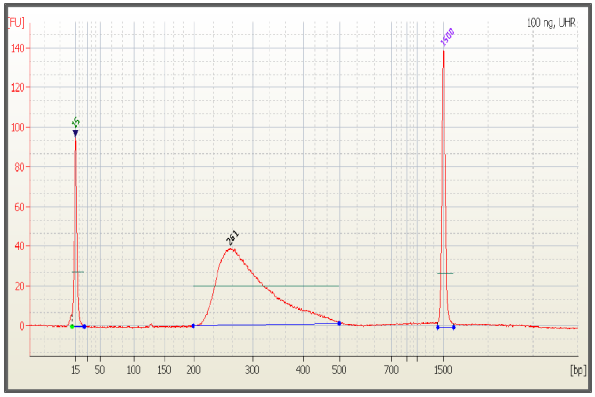
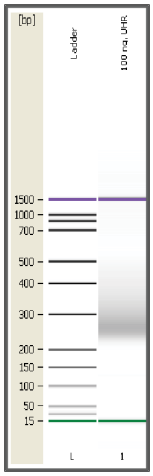


Figure 40 TruSeq Stranded Total RNA Sample Prep 260 bp PCR Product



Normalize and Pool Libraries

This process describes how to prepare DNA templates that will be applied to 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 indexing are normalized to 10 nM in the DCT plate without pooling.

Consumables

Item	Quantity	Storage	Supplied By
DCT (Diluted Cluster Template) barcode label	1 label per plate	15° to 30°C	Illumina
PDP (Pooled DCT Plate) barcode label (for indexing only)	1 label per plate	15° to 30°C	Illumina
96-well 0.3 ml PCR plate (for indexing only)	1	15° to 30°C	User
96-well MIDI plate	1	15° to 30°C	User
Microseal 'B' Adhesive Seals	2	15° to 30°C	User
Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20	Enough to normalize the concentration of each sample library to 10 nM	15° to 30°C	User

Preparation

- Apply a DCT barcode label to a new 96-well MIDI plate.
- Apply a PDP barcode label to a new 96-well 0.3 ml PCR plate (for indexing only).
- Remove the TSP1 plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 91, and let stand to thaw at room temperature.
  - Centrifuge the thawed TSP1 plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.

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 DCT plate to 10 nM using Tris-Cl 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-indexed libraries, the protocol stops here. Do one of the following:
     - Proceed to cluster generation. For more information, see the *Illumina Cluster Generation User Guide*.
     - Seal the DCT plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.
   - For indexed libraries, proceed to Make PDP.

Make PDP (for indexing only)

NOTE
Do not make a PDP plate if there is no pooling.

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

   NOTE
   Keep track 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 µl 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 should be 10X the number of combined sample libraries and will be 20–240 µl (2–24 libraries). For example, the volume for 2 samples is 20 µl, the volume for 12 samples is 120 µl, or the volume for 24 samples is 240 µl.

- If pooling 25–96 samples:
  - Using a multichannel pipette, transfer 5 µl of each normalized sample library in column 1 from the DCT plate to column 1 of the new MIDI plate labeled with the PDP barcode.
  - Transfer 5 µl 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 will be 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 *Illumina Cluster Generation User Guide*.
- Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.

High Sample (HS) Protocol

Chapter 4

Introduction 100
Sample Prep Workflow 102
Ribo-Zero Deplete and Fragment RNA 103
Synthesize First Strand cDNA 112
Synthesize Second Strand cDNA 115
Adenylate 3' Ends 119
Ligate Adapters 122
Enrich DNA Fragments 129
Validate Library 134
Normalize and Pool Libraries 136

Introduction

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

Table 23 Kit and Sample Number Recommendations

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

Table 24 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

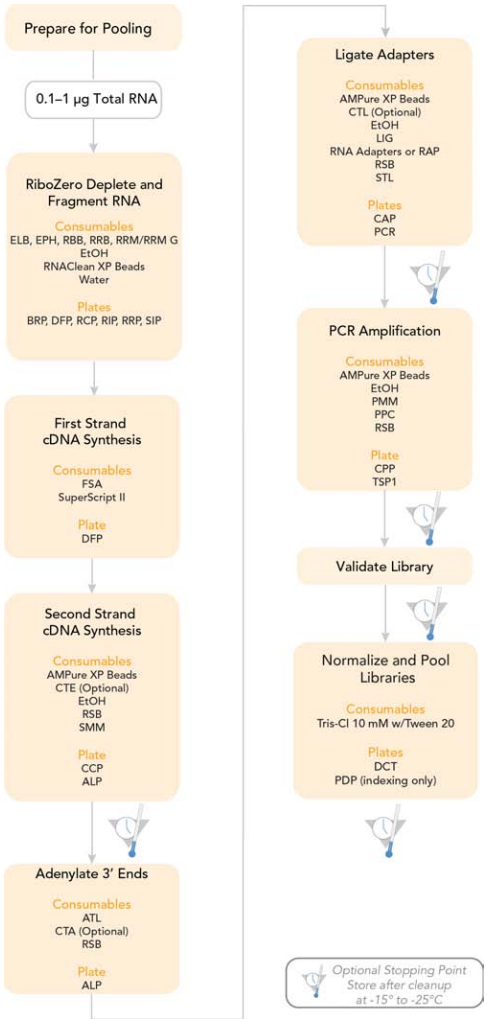
- Review *Best Practices* on page 12 before proceeding.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- This HS protocol requires shaking and heating equipment to mix reagents and for incubation (see *Consumables and Equipment* on page 40).

- For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample preparation. For more information, see *Tracking Tools* on page 29.
- If you are pooling using adapter index tubes, record information about your samples before beginning library preparation for later use in data analysis. For more information, see *Tracking Tools* on page 29. Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
- If you are pooling with the RAP, review the planning steps in *Pooling Preparation with Adapter Plate* on page 47 before beginning library preparation.

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 41 TruSeq Stranded Total RNA Sample Preparation HS Workflow

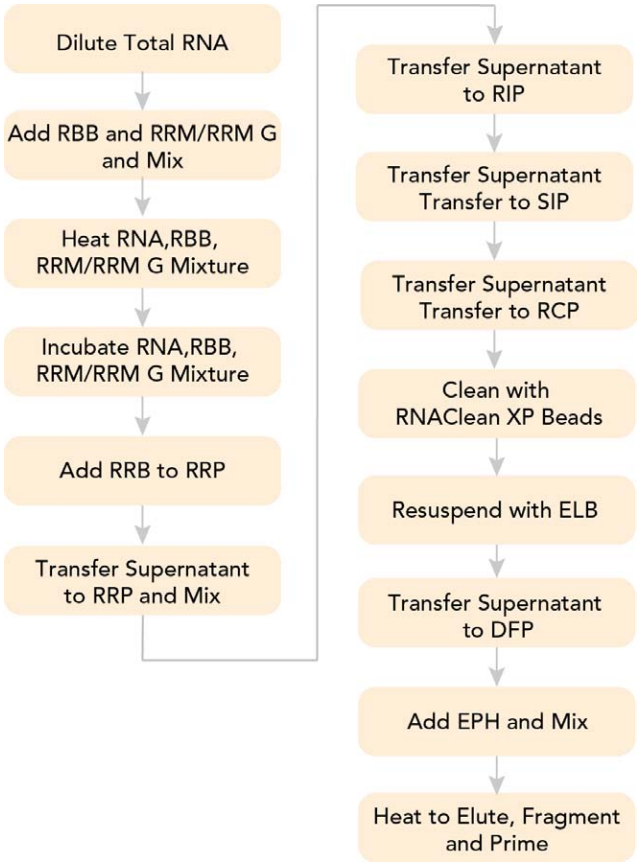


Ribo-Zero Deplete and Fragment RNA

This process depletes ribosomal RNA from total RNA. After the ribosomal RNA is depleted, the remaining RNA is purified, fragmented and primed for cDNA synthesis.

Reference the following diagram while performing the purification procedures:

Figure 42 TruSeq Stranded Total RNA Sample Prep Purification Workflow



It is important to follow this procedure exactly to be sure of reproducibility.

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
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.

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° to -25°C	Illumina
Elution Buffer (ELB)	1 tube per 48 reactions	2° to 8°C	Illumina
One of the following, depending on the kit you are using: • Ribo-Zero rRNA Removal Mix (RRM) (Ribo-Zero Human/Mouse/Rat kit contents) • Ribo-Zero rRNA Removal Mix - Gold (RRM G) (Ribo-Zero Gold kit contents)	1 tube per 48 reactions	-15° to -25°C	Illumina

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	-15° to -25°C	Illumina
rRNA Binding Buffer (RBB)	1 tube per 48 reactions	2° to 8°C	Illumina
rRNA Removal Beads (RRB)	1 tube per 48 reactions	2° to 8°C	Illumina
BRP (Bind rRNA Plate) barcode label	1 label per plate	15° to 30°C	Illumina
DFP (Depleted RNA Fragmentation Plate) barcode label	1 label per plate	15° to 30°C	Illumina
RCP (RNA Clean Up Plate) barcode label	1 label per plate	15° to 30°C	Illumina
RIP (Removal Intermediate Plate) barcode label	1 label per plate	15° to 30°C	Illumina
RRP (rRNA Removal Plate) barcode label	1 label per plate	15° to 30°C	Illumina
SIP (Second Removal Intermediate Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well HSP plates	2	15° to 30°C	User
96-well MIDI plates	4	15° to 30°C	User
Freshly Prepared 70% Ethanol (EtOH)	200 µl per sample	15° to 30°C	User
Microseal 'B' Adhesive Seals	5	15° to 30°C	User
RNAClean XP Beads	99 µl per sample	2° to 8°C	User

Item	Quantity	Storage	Supplied By
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	6	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	6	15° to 30°C	User
Ultra Pure Water	Enough to dilute each total RNA sample to a final volume of 10 µl	15° to 30°C	User

Preparation

- Remove the following from -15° 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:
    - Ribo-Zero rRNA Removal Mix
    - Ribo-Zero rRNA Removal Mix - Gold
  - Resuspension Buffer

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

- Remove the following from 2° to 8°C storage and let stand to bring to room temperature:
  - Elution Buffer
  - rRNA Binding Buffer
  - rRNA Removal Beads
- Remove the RNAClean XP beads from 2° 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
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° 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 a RCP barcode label to a new 96-well MIDI plate.
- Apply a RIP barcode label to a new 96-well MIDI plate.
- Apply a RRP barcode label to a new 96-well MIDI plate.
- Apply a SIP 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. Do one of the following, depending on the kit you are using:
   - Add 5 µl of Ribo-Zero rRNA Removal Mix to each well of the BRP plate.
   - Add 5 µl of Ribo-Zero rRNA Removal Mix - Gold to each well of the BRP plate.
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 1,600 rpm for 20 seconds.
   c. Centrifuge the BRP plate to 280 xg for 1 minute.
5. Return the rRNA Binding Buffer to 2° to 8°C storage.
6. Do one of the following, depending on the kit you are using:
   - Return the Ribo-Zero rRNA Removal Mix to -15° to -25°C storage.
   - Return the Ribo-Zero rRNA Removal Mix - Gold to -15° to -25°C storage.

Incubate 1 BRP

1. Place the sealed BRP plate on the pre-programmed thermal cycler. Close the lid and select **RNA Denaturation** to denature the RNA.
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 completely resuspend the beads.
2. Add 35 µl of rRNA Removal Beads to each well of the new 96-well MIDI plate labeled with the RRP barcode.

   NOTE
   It is important to not 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 and transfer the entire contents (20 µl) from each well of the BRP plate to the corresponding well of the RRP plate containing rRNA Removal Beads.
4. 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 1,000 rpm for 1 minute.
5. Place the RRP plate on the magnetic stand at room temperature for 1 minute.
6. Remove the adhesive seal from the RRP plate.
7. 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 RIP barcode.
8. Place the RIP plate on the magnetic stand at room temperature for 1 minute.
9. Transfer all of the supernatant from each well of the RIP plate to the corresponding well of the new 96-well MIDI plate labeled with the SIP barcode.
10. Place the SIP plate on the magnetic stand at room temperature for 1 minute.

11 Transfer all of the supernatant from each well of the SIP plate to the corresponding well of the new 96-well MIDI plate labeled with the RCP barcode.

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.

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

Clean Up RCP

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 14 when working with RNAClean XP beads.

1 Vortex the RNAClean XP beads until they are well dispersed, then add 99 µl 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 µl 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 1,800 rpm for 2 minutes.

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 the adhesive seal from the RCP plate.

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 remaining on the magnetic stand, add 200 µl of freshly prepared 70% EtOH to each well without disturbing the beads.

7 Incubate the RCP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

8 Let the 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 xg for 5 seconds.

10 Add 11 µl 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 1,800 rpm for 2 minutes.
   c Centrifuge the RCP plate to 280 xg for 1 minute.

11 Incubate the RCP plate at room temperature for 2 minutes.

12 Place the RCP plate on the magnetic stand at room temperature for 5 minutes.

13 Return the Elution Buffer to 2° to 8°C storage.

14 Remove the adhesive seal from the RCP plate.

15 Transfer 8.5 µl of the supernatant from the RCP plate to the new 96-well HSP plate labeled with the DFP barcode.

16 Add 8.5 µl 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 1,600 rpm for 20 seconds.

17 Return the Elute, Prime, Fragment High Mix to -15° to -25°C storage and the RNAClean XP Beads tube to 2° 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.

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 112.

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.

Consumables

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

WARNING

First Strand Synthesis Mix Act D (FSA) 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. Please 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 http://www.illumina.com/msds.

Preparation

- Remove one tube of First Strand Synthesis Act D Mix from -15° 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 1,000 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° 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 xg 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 1,600 rpm for 20 seconds.

5 Return the First Strand Synthesis Mix Act D tube to -15° 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**.

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 115.

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. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix. At the end of this process, you will have blunt-ended cDNA.

Consumables

Item	Quantity	Storage	Supplied By
(Optional) End Repair Control (CTE)	1 tube per 48 reactions	2° to 8°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Second Strand Marking Master Mix (SMM)	1 tube per 48 reactions	-15° to -25°C	Illumina
ALP (Adapter Ligation Plate) barcode label	1 label per plate	15° to 30°C	Illumina
CCP (cDNA Clean Up Plate) barcode label	1 label per plate	15° to 30°C	Illumina
IMP (Insert Modification Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well MIDI plates	2	15° to 30°C	User
AMPure XP beads	90 µl per sample	2° to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	15° to 30°C	User
Microseal 'B' Adhesive Seals	4	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	5	15° to 30°C	User

Item	Quantity	Storage	Supplied By
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	5	15° to 30°C	User

Preparation

- Remove the following from -15° 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° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 14.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the thermal cycler to 16°C.
- Choose the thermal cycler pre-heat lid option and set to 30°C
- Apply a 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 xg 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 xg for 5 seconds.
4. Add 20 µl 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 1,600 rpm for 20 seconds.
5. Return the Second Strand Marking Master Mix tube to -15° 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, remove the adhesive seal, and let stand to bring the plate to room temperature.

Clean Up DFP

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 14 when working with AMPure XP Beads.

1. Vortex the AMPure XP beads until they are well dispersed, then add 90 µl of well-mixed AMPure XP beads to each well of the new MIDI plate labeled with the CCP barcode.
2. 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 1,800 rpm for 2 minutes.
3. Incubate the CCP plate at room temperature for 15 minutes.
4. 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.

5 Remove the adhesive seal from the CCP plate.

6 Remove and discard 135 µl of the 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 (7–9).

7 With the CCP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

8 Incubate the CCP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

9 Repeat steps 7 and 8 once for a total of two 80% EtOH washes.

10 Let the plate stand at room temperature for 15 minutes to dry and then remove the CCP plate from the magnetic stand.

11 Centrifuge the thawed, room temperature Resuspension Buffer to 600 xg for 5 seconds.

12 Add 17.5 µl 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 1,800 rpm for 2 minutes.

c Centrifuge the CCP plate to 280 xg for 1 minute.

13 Incubate the CCP plate at room temperature for 2 minutes.

14 Place the CCP plate on the magnetic stand at room temperature for 5 minutes.

15 Remove the adhesive seal from the CCP plate.

16 Transfer 15 µl of the supernatant (ds cDNA) from the CCP plate to the new MIDI plate labeled with the ALP barcode.

SAFE STOPPING POINT
If you do not plan to proceed to *Adenylate 3' Ends* on page 119 immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven 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° to -25°C	Illumina
A-Tailing Mix (ATL)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Microseal 'B' Adhesive Seal	1	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	3	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	3	15° to 30°C	User

Preparation

- Remove the following from -15° 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° to 8°C storage and bring it to room temperature.
- Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up DFP* on page 117 and let stand to thaw at room temperature.
  - Centrifuge the thawed ALP plate to 280 xg 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.

Add ATL

1. Do one of the following:
   - If using the in-line control reagent:
     - Centrifuge the thawed A-Tailing Control tube to 600 xg 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 1,800 rpm for 2 minutes.
   c. Centrifuge the ALP plate to 280 xg 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 122.

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° to -25°C	Illumina

Choose from the following depending on the kit you are using:
• TruSeq Stranded Total RNA LT Sample Prep Kit contents:
  • RNA Adapter Indices (AR001–AR016, AR018–AR023, AR025, AR027)
• TruSeq Stranded Total RNA HT Sample Prep Kit contents:
  • RAP (RNA Adapter Plate)
	1 tube per column of 8 reactions, of each indices being used
	or
	1 RAP
		-15° to -25°C	Illumina

Ligation Mix (LIG)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
Stop Ligation Buffer (STL)	1 tube per 48 reactions	-15° to -25°C	Illumina
CAP (Clean Up ALP Plate) barcode label	1 label per plate	15° to 30°C	Illumina
PCR (Polymerase Chain Reaction) barcode label	1 label per plate	15° to 30°C	Illumina

Item	Quantity	Storage	Supplied By
RAP (RNA Adapter Plate) barcode label (if using the HT kit)	1 label per plate	15° to 30°C	Illumina
96-well HSP plate	1	15° to 30°C	User
96-well MIDI plate	1	15° to 30°C	User
AMPure XP beads	92 µl per sample	2° to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)	800 µl per sample	15° to 30°C	User
Microseal 'B' Adhesive Seals	7	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	4–28	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	4–28	15° to 30°C	User

Preparation

- Remove the following from -15° 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.
    - If using the RAP, review *Handling Adapter Plate* on page 48.
  - Stop Ligation Buffer

    NOTE
    Do not remove the Ligation Mix tube from -15° 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° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 14.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- 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.

NOTE

- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate 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

Illumina recommends that the RAP does not undergo more than 4 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.

Add LIG

1 Do one of the following:
- If using RNA Adapter tubes, centrifuge the appropriate/desired thawed tubes to 600 xg for 5 seconds.
- If using a RAP:
  - Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to ensure that they all are completely thawed.
  - Remove the adapter plate tape seal.
  - Centrifuge the plate at 280 xg for 1 minute to collect all of the adapter to the bottom of the well.
  - Remove the plastic cover and save the cover if you are not processing the entire plate at once.
  - If this 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 xg for 5 seconds.

3 Immediately before use, remove the Ligation Mix tube from -15° to -25°C storage.

4 Remove the adhesive seal from the ALP plate.

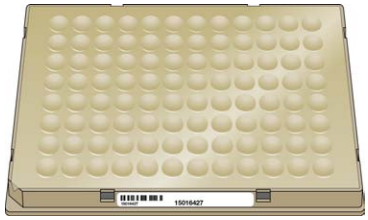
5 Do one of the following:
- If using the in-line control reagent:
  - Dilute the Ligation Control to 1/100 in Resuspension Buffer (For example, 1 µl Ligation Control + 99 µl Resuspension Buffer) before use. Discard the diluted Ligation Control after use.
  - Add 2.5 µl of diluted Ligation Control to 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.

6 Add 2.5 µl of Ligation Mix to each well of the ALP plate.

7 Return the Ligation Mix tube back to -15° to -25°C storage immediately after use.

8 Do one of the following:
- If using RNA Adapter tubes, add 2.5 µl of the appropriate/desired 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 located on the lower left.

Figure 43 Correct RAP Orientation



  - Do one of the following to pierce the foil seal:
    - If using the entire plate at once, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously by gently but firmly pressing 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 desired columns that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each column of adapters that will be used for ligation.
- Using an 8-tip multichannel pipette, transfer 2.5 µl of the appropriate/desired 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 1,800 rpm for 2 minutes.
   c Centrifuge the ALP plate to 280 xg 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 µl 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 1,800 rpm for 2 minutes.

Clean Up ALP

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 14 when working with AMPure XP Beads.

1 Remove the adhesive seal from the ALP plate.

2 Vortex the AMPure XP Beads until they are well dispersed, then add 42 µl 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 1,800 rpm for 2 minutes.

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 appears clear.

5 Remove the adhesive seal from the ALP plate.

6 Remove and discard 79.5 µl of the supernatant from each well of the ALP plate.

NOTE
Leave the ALP plate on the magnetic stand while performing the following 80% EtOH wash steps (7–9).

7 With the ALP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

8 Incubate the ALP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

9 Repeat steps 7 and 8 once for a total of two 80% EtOH washes.

10 While keeping the ALP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes.

11 Resuspend the dried pellet in each well with 52.5 µl Resuspension Buffer. 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 1,800 rpm for 2 minutes.

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 appears clear.

14 Remove the adhesive seal from the ALP plate.

15 Transfer 50 µl of the clear supernatant from each well of the ALP plate to the corresponding well of the new MIDI plate labeled with the CAP barcode.

16 Vortex the AMPure XP Beads until they are well dispersed, then add 50 µl of mixed AMPure XP Beads 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 1,800 rpm for 2 minutes.

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 appears clear.

19 Remove the adhesive seal from the CAP plate.

20 Remove and discard 95 µl of the supernatant from each well of the CAP plate.

NOTE
Leave the CAP plate on the magnetic stand while performing the following 80% EtOH wash steps (21–23)

21 With the CAP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

22 Incubate the CAP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

23 Repeat steps 21 and 22 once for a total of two 80% EtOH washes.

24 While keeping the CAP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes.

25 Resuspend the dried pellet in each well with 22.5 µl Resuspension Buffer. 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 1,800 rpm for 2 minutes.

26 Incubate the CAP plate at room temperature for 2 minutes.

27 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

28 Remove the adhesive seal from the CAP plate.

29 Transfer 20 µl of the clear supernatant from each well of the CAP plate to the corresponding well of the new HSP plate labeled with the PCR barcode.

SAFE STOPPING POINT
If you do not plan to proceed to *Enrich DNA Fragments* on page 129 immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -15° 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. The number of PCR cycles should be minimized 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, as fragments without any adapters cannot hybridize to surface-bound primers in the flow cell, and 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° to -25°C	Illumina
PCR Primer Cocktail (PPC)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
CPP (Clean Up PCR Plate) barcode label	1 label per plate	15° to 30°C	Illumina
TSP1 (Target Sample Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well HSP plate	1	15° to 30°C	User
96-well MIDI plate	1	15° to 30°C	User
AMPure XP beads	50 µl per sample	2° to 8°C	User

Item	Quantity	Storage	Supplied By
Freshly Prepared 80% Ethanol (EtOH)	400 µl per sample	15° to 30°C	User
Microseal 'A' Film	1	15° to 30°C	User
Microseal 'B' Adhesive Seals	3	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	5	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	5	15° to 30°C	User

Preparation

- Remove the PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature.
- Centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- Remove the Resuspension Buffer from 2° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 14.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the PCR plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 126 and let stand to thaw at room temperature.
  - Centrifuge the thawed PCR plate to 280 xg 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 HSP 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. Mix thoroughly as follows:
   a. Seal the PCR plate with a Microseal 'A' film.

WARNING
Follow the vendor's 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 1,600 rpm for 20 seconds.
   c. Centrifuge the PCR plate to 280 xg for 1 minute.

Amp PCR

1. Place the sealed PCR plate on the pre-programmed thermal cycler. Close the lid and select **PCR** to amplify the plate.

Clean Up PCR

NOTE
Before performing clean up, review *Handling Magnetic Beads* on page 14 when working with AMPure XP Beads.

1. Remove the adhesive seal from the PCR plate.
2. Vortex the AMPure XP Beads until they are well dispersed, then do one of the following:
   - If using the RNA Adapter tubes, add 50 µl 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 µl of the mixed AMPure XP Beads to each well of the new MIDI plate labeled with the CPP barcode.

3 Transfer the entire contents from each well of the PCR plate to the corresponding well of the CPP plate containing 50 µl 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 1,800 rpm for 2 minutes.

4 Incubate the CPP plate at room temperature for 15 minutes.

5 Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

6 Remove the adhesive seal from the CPP plate.

7 Remove and discard 95 µl of the 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 (8–10).

8 With the CPP plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

9 Incubate the CPP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well.

10 Repeat steps 8 and 9 once for a total of two 80% EtOH washes.

11 While keeping the CPP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes.

12 Resuspend the dried pellet in each well with 32.5 µl Resuspension Buffer. 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 1,800 rpm for 2 minutes.

13 Incubate the CPP plate at room temperature for 2 minutes.

14 Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

15 Remove the adhesive seal from the CPP plate.

16 Transfer 30 µl of the clear supernatant from each well of the CPP plate to the corresponding well of the new HSP plate labeled with the TSP1 barcode.

SAFE STOPPING POINT

If you do not plan to proceed to *Validate Library* on page 134 immediately, the protocol can be safely stopped here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven 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

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

Quality Control

1. Load 1 µl 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 44 Example of TruSeq Stranded Total RNA Sample Prep Library Size Distribution

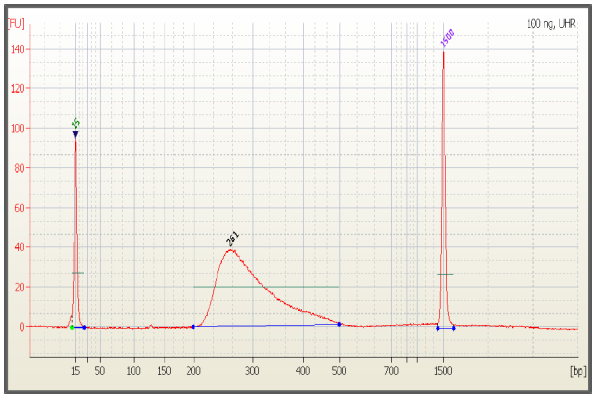
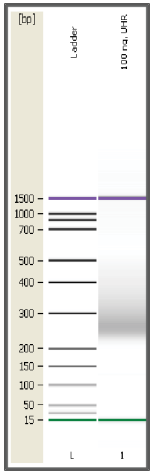


Figure 45 TruSeq Stranded Total RNA Sample Prep 260 bp PCR Product



Normalize and Pool Libraries

This process describes how to prepare DNA templates that will be applied to 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 indexing are normalized to 10 nM in the DCT plate without pooling.

Consumables

Item	Quantity	Storage	Supplied By
DCT (Diluted Cluster Template) barcode label	1 label per plate	15° to 30°C	Illumina
PDP (Pooled DCT Plate) barcode label (for indexing only)	1 label per plate	15° to 30°C	Illumina
96-well HSP plate (for indexing only)	1	15° to 30°C	User
96-well MIDI plate	1	15° to 30°C	User
Microseal 'B' Adhesive Seals	5	15° to 30°C	User
Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20	Enough to normalize the concentration of each sample library to 10 nM	15° to 30°C	User

Preparation

- Apply a DCT barcode label to a new 96-well MIDI plate.
- Apply a PDP barcode label to a new 96-well HSP plate (for indexing only).
- Remove the TSP1 plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 131, and let stand to thaw at room temperature.
  - Centrifuge the thawed TSP1 plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.

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 DCT plate to 10 nM using Tris-Cl 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 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 1,000 rpm for 2 minutes.

c Centrifuge the DCT plate to 280 xg for 1 minute.

d Remove the adhesive seal from the DCT plate.

4 Depending on the type of library you want to generate, do one of the following:

- For non-indexed libraries, the protocol stops here. Do one of the following:
  - Proceed to cluster generation. For more information, see the *Illumina Cluster Generation User Guide*.
  - Seal the DCT plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.
- For indexed libraries, proceed to Make PDP.

Make PDP (for indexing only)

NOTE
Do not make a PDP plate if there is no pooling.

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

NOTE
Keep track 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 µl 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 should be 10X the number of combined sample libraries and will be 20–240 µl (2–24 libraries). For example, the volume for 2 samples is 20 µl, the volume for 12 samples is 120 µl, or the volume for 24 samples is 240 µl.

- If pooling 25–96 samples:
  - Using a multichannel pipette, transfer 5 µl of each normalized sample library in column 1 from the DCT plate to column 1 of the new MIDI labeled with the PDP barcode.
  - Transfer 5 µl 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 will be a PDP plate with pooled samples in column 1. Mix the PDP plate as follows:
    - Seal PDP plate with Microseal 'B' adhesive seal.
    - Shake PDP plate on microplate shaker at 1,800 rpm for 2 minutes.
  - 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 1,800 rpm for 2 minutes.
4. Do one of the following:
   - Proceed to cluster generation. For more information, see the *Illumina Cluster Generation User Guide*.
   - Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.

Alternate Fragmentation Protocols

Appendix A

Introduction 140
Modify RNA Fragmentation Time for Intact RNA 141
Modify RNA Fragmentation Time for Degraded RNA 143

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 Sample Prep fragmentation protocol for transcriptome analysis is performed on the RNA after rRNA depletion using elevated temperatures, resulting in libraries with inserts ranging in size from 120-200 bp with a median size of 150 bp. The TruSeq Stranded Total RNA Sample Prep 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 141.

Illumina also recognizes that it is not always possible to extract intact total RNA. For instance, RNA extracted from FFPE samples is usually degraded. To vary the fragmentation time for degraded RNA, see *Modify RNA Fragmentation Time for Degraded RNA* on page 143.

Modify RNA Fragmentation Time for Intact RNA

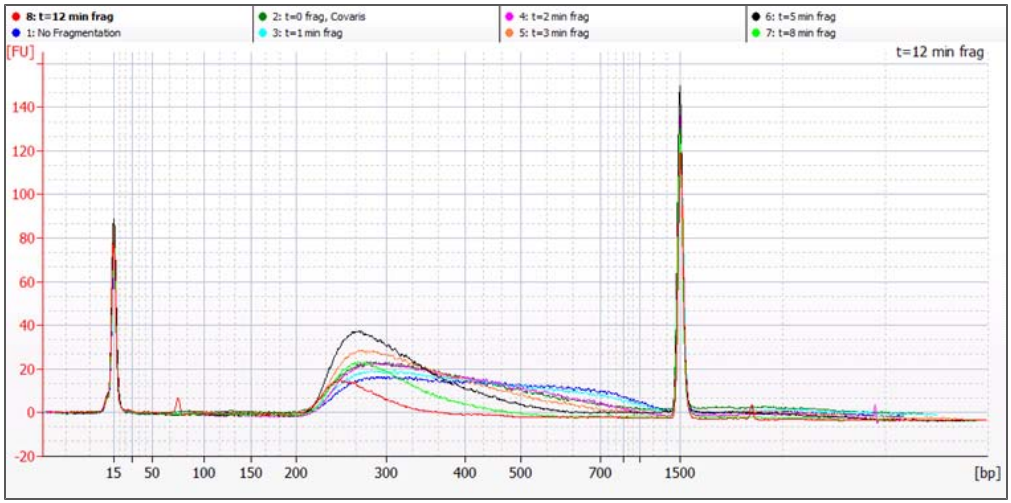
To modify the fragmentation of the RNA to allow for longer RNA fragments, the time of fragmentation can be shortened. This is accomplished during the *Ribo-Zero Deplete and Fragment RNA* procedures by modifying the thermal cycler **Elution 2 - Frag - Prime** program: 94°C for X minutes followed by a 4°C hold for the thermal cycler where X is determined by the length of RNA desired. A range of suggested times and sizes is described in Table 25.

Table 25 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. Start without bringing up to temperature.

Figure 46 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 altogether (*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 47—Figure 51, 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 51, no fragmentation is necessary. Skip *Incubate 1 DFP* and proceed immediately to *Synthesize First Strand cDNA*.

Figure 47 Incubate Samples at 94°C for 8 Minutes, Followed By a 4°C Hold

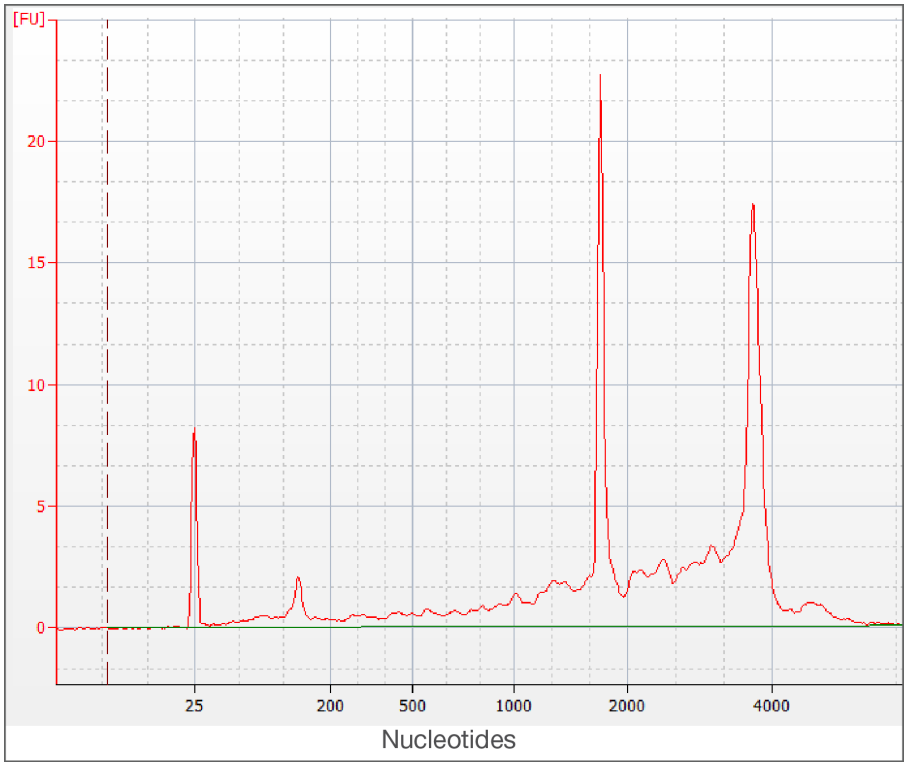


Figure 48 Incubate Samples at 94°C for 7 Minutes, Followed By a 4°C Hold

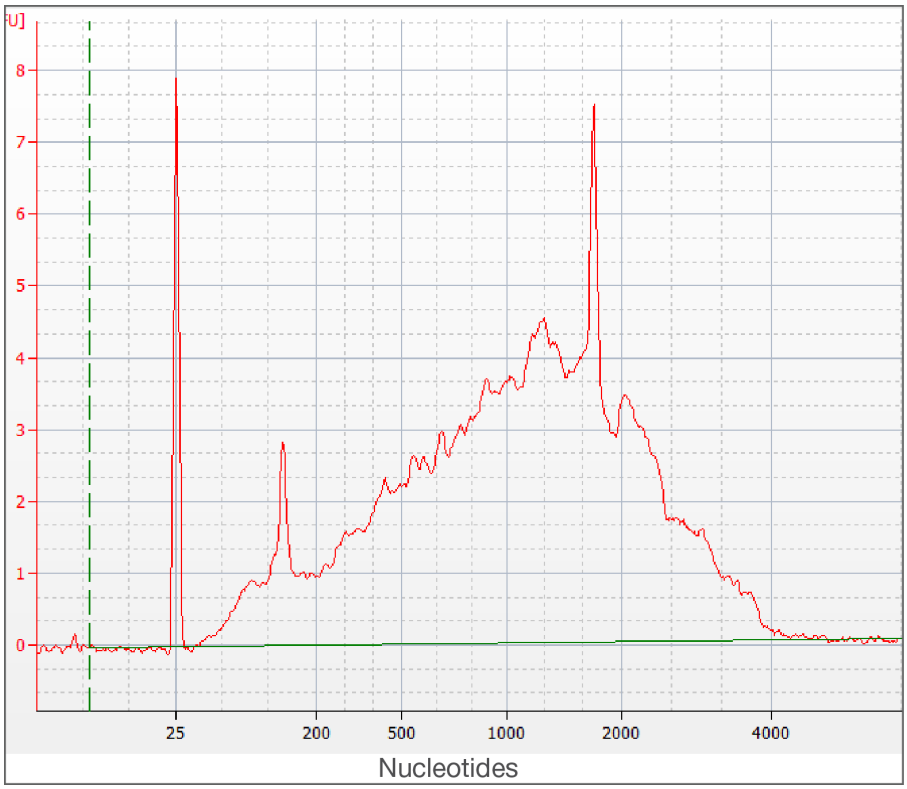


Figure 49 Incubate Samples at 94°C for 6 Minutes, Followed By a 4°C Hold

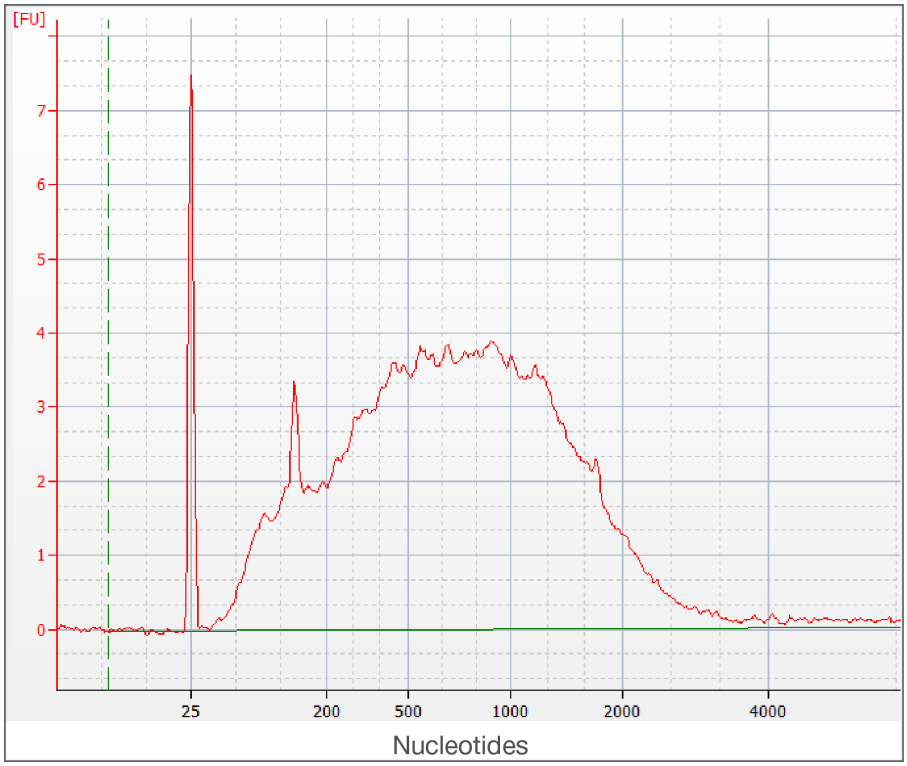


Figure 50 Incubate Samples at 94°C for 4 Minutes, Followed By a 4°C Hold

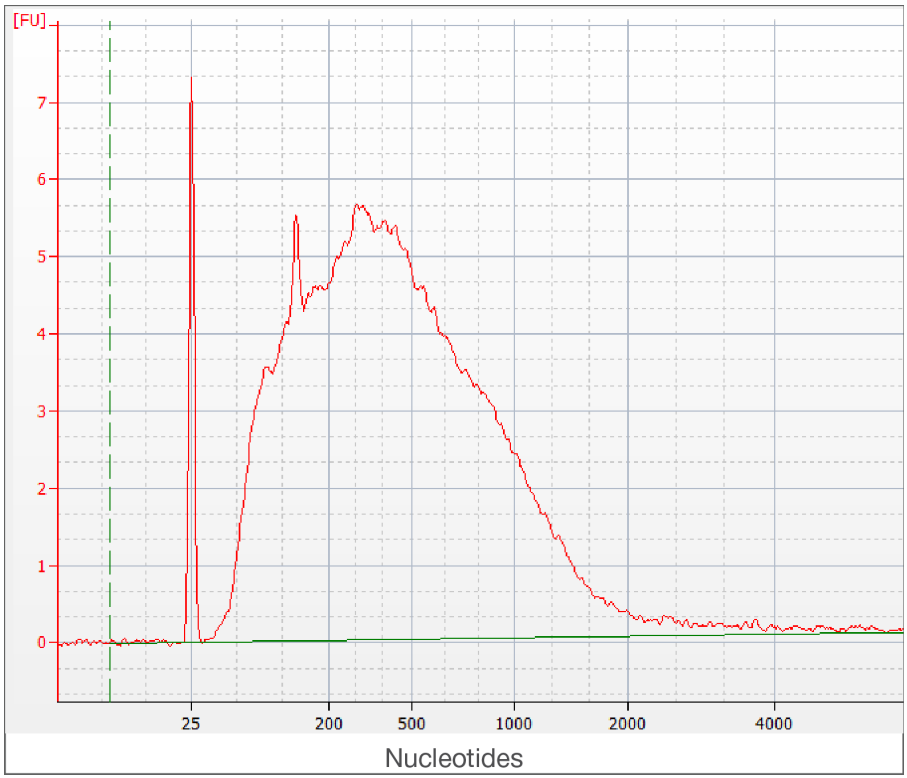
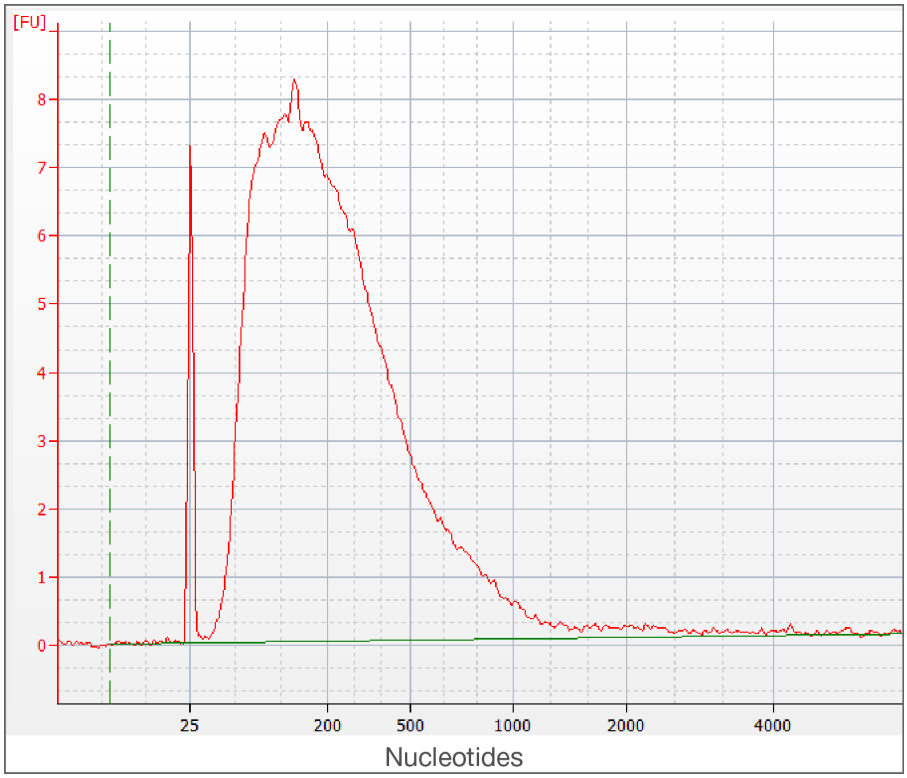


Figure 51 No Fragmentation Necessary. Skip *Incubate 1 DFP* and Proceed to *Synthesize First Strand cDNA*



Index

A

Acronyms 9
Actinomycin D 74
adapter plate iii
Add ATL 81, 120
Add LIG 84-85
Add SMM 77, 116
Add STL 86, 126
Agilent Bioanalyzer 13
ALP 76, 115
Amp PCR 91, 131
AMPure XP Beads 14, 76, 83, 89, 115, 123, 129
ATL 80, 119

B

BRB 67, 105

C

CAP 82, 122
CCP 115
cDNA synthesis 65, 103
Clean Up ALP 86, 126
Clean Up DFP 78, 117
Clean Up PCR 91, 131
Clean Up RCP 71, 109
cluster generation 2, 97, 138
CPP 129
cross-contamination 12, 15
CTA 80, 119
CTE 76, 115
CTL 82, 122
customer support 153

D

DCT 95, 136
degradation 12
degraded RNA 66, 71, 104, 109, 140, 143
DFP 67, 105
documentation 153
ds cDNA 12, 16, 76, 115

E

ELB 66, 104
Elution 2 - Frag - Prime 141
EPH 66, 104
experienced user card (EUC) iii, 29
Experiment Manager 29, 51-52, 56

F

FFPE 66, 71, 104, 109, 140, 143
first strand cDNA 2
fragmentation time 140
FSA 73, 112

H

handling RNA 12
harvesting 12
help, technical 153
High Sample (HS) 4
High Throughput (HT) iii
HSP 4

I

IMP 115

in-line control DNA 27
Incubate 1 ALP 81, 120
Incubate 1 BRP 69, 108
Incubate 1 DFP 72, 110
Incubate 2 ALP 86, 126
Incubate 2 DFP 75, 114
Incubate 3 DFP 78, 117
index adapter 2
index sequences iii
indexed adapter 43-44

K

kit options iii

L

lab tracking form (LTF) 29
Lab Tracking Form (LTF) iii
LIG 82, 122
liquid handling 13
Low Sample (LS) 4

M

magnetic beads 14
Make BRP 69, 107
Make DCT 96, 137
Make PCR 91, 131
Make PDP 96, 137
Make RRP iii, 70, 108
micro plate shaker 4
microheating system 4
MIDI 4
mRNA Denaturation 69, 108

P

PCR 3, 82, 122
PDP 95, 136
PicoGreen 13
PMM 89, 129
pooled sample volumes 97, 138
pooling iii, 47-48, 51
PPC 89, 129

Q

qPCR 13
quality control 93, 134
quantify libraries 93, 134

R

RAP iii, 15, 17, 47-48, 82, 122
RBB 67, 105
RCP 67, 105
Reagent Reservoirs 67, 73, 76, 80, 83, 90, 106, 112, 115, 119, 123, 130
Ribo-Zero 2, 30, 34, 65-66, 69, 103-104, 107
RIP 67, 105
RNA Adapter Indices 82, 122
RNAClean XP Beads 14, 67, 105
RNAse-free 12
RRB 67, 105
RRM 66, 104
RRM G 66, 104
RRP 67, 105
RSB iii, 67, 76, 80, 82, 89, 105, 115, 119, 122, 129

S

SAV 27-28
second strand cDNA 2
SIP 67, 105
SMM 76, 115
STL 82, 122
strip tubes and caps 68, 73, 76, 80, 83, 90, 106, 112, 116, 119, 123, 130
SuperScript II 73, 112

T

technical assistance 153
temperature 15

thermal cycler 4, 24
total RNA 2, 12
Tris-Cl 95, 136
TSP1 89, 95, 129, 136

U

ultra pure water 68, 106
Usage Guidelines iii, 16

W

workflow diagram 64, 102

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 26 Illumina General Contact Information

Illumina Website	http://www.illumina.com
Email	techsupport@illumina.com

Table 27 Illumina Customer Support Telephone Numbers

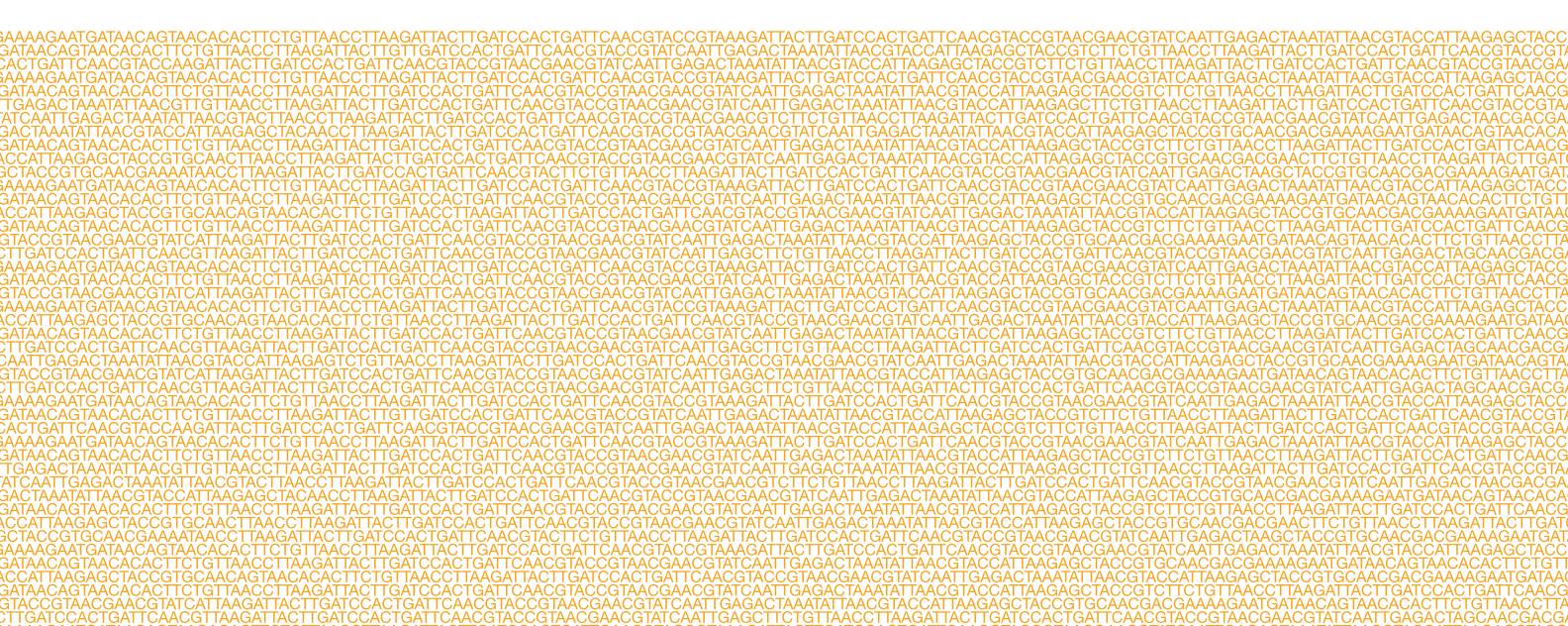
Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	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 http://www.illumina.com/msds.

Product Documentation

You can obtain PDFs of additional product documentation from the Illumina website. Go to http://www.illumina.com/support and select a product. To download documentation, you will be asked to log in to MyIllumina. After you log in, you can view or save the PDF. To register for a MyIllumina account, please visit https://my.illumina.com/Account/Register.



Illumina
Headquartered in San Diego, California, U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com