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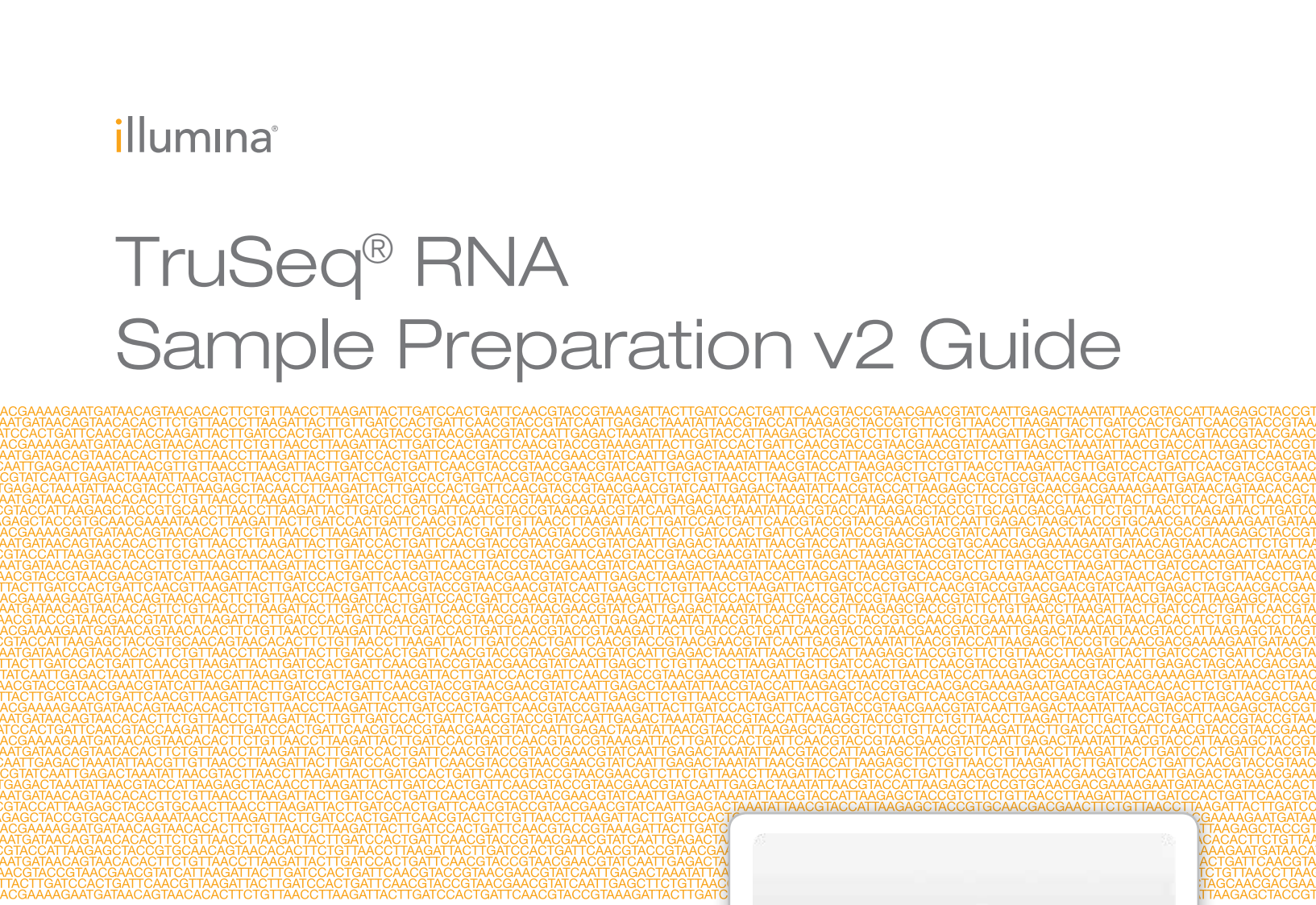
TruSeq® RNA Sample Preparation v2 Guide



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Revision History

Part #	Revision	Date	Description of Change
15026495	F	March 2014	• Corrected 42°C incubation time in Incubate 1 CDP procedures
15026495	E	January 2014	• Modified total RNA input to 0.1–1 µg • Modified alternative isolated mRNA input to 10–100 ng • Renamed Incubate 1 IMP to Incubate IMP • Corrected *Kit Contents* box 1 shipping temperature • Corrected the RNA Purification Beads part number • Added bioanalyzer and DNA 1000 Kit to equipment list • Created new section of *Additional Resources* • Added reference to BaseSpace® to organize samples, libraries, pools, and runs • Replaced *Best Practices* section with a reference to content on the Illumina website • Replaced *Adapter Options* and *Pooling Guidelines* sections with a reference to the *TruSeq Sample Preparation Pooling Guide (part # 15042173)* • Created new appendix of *Supporting Information* containing *Acronyms*, *Kit Contents*, *Consumables and Equipment*, and *Indexed Adapter Sequences* • In the *Alternate Fragmentation Protocols* Appendix, clarified instructions for samples requiring 0 minutes fragmentation time

Part #	Revision	Date	Description of Change

15026495	D	September 2012	• Clarified that when starting with previously isolated mRNA, begin the protocol at the Incubate RFP procedures
			• Revised *Usage Guidelines* reagent volume table :
			  • Added RNA Adapter tubes and RNA Purification Beads
			  • Revised the control, Bead Washing Buffer and First Strand Master Mix volumes
			  • Removed Resuspension Buffer because it varies per procedure
			• Corrected PCR Primer Cocktail part number in Kit Contents
			• Reformatted the consumables list at the start of each procedure to a table.
			• Modified Purify and Fragment mRNA preparation to include thawing Resuspension Buffer to allow adequate time before use during Synthesize Second Strand cDNA procedure. Removed instructions to thaw Resuspension Buffer during Perform End Repair preparation, because it has already been thawed.
			• After initial thaw, modified Resuspension Buffer storage to be to 2°C to 8°C
			• Modified Incubate 1 ALP procedures during Adenylate 3' Ends process to add an incubation at 70°C for 5 minutes followed by a 4°C hold.
			• Added recommendation for two microheating systems and MIDI plate inserts to support modified Incubate 1 ALP procedures in HS protocol

Part #	Revision	Date	Description of Change

15026495	C	May 2012	• Added reagent volume table to *Usage Guidelines*
			• Revised *Tracking Tools* documentation download information
			• Added kit box reference for each indexed adapter
			• Added *Pooling Guidelines* for low-plexity index combinations
			• Changed *Consumables and Equipment* supplier name for 1.5 ml RNase/DNase-free non-sticky tubes and magnetic stand-96
			• Revised sample prep workflow diagrams to include Perform End Repair procedure
			• Removed instructions throughout LS protocol to adjust pipette to a specified volume before mixing.
			• Make RFP - added step to centrifuge BBB before adding to samples
			• Make CDP - Clarified SuperScript II to First Strand Master Mix tube ratio
			• *Appendix A - Alternate Fragmentation Protocol*:
			  • Clarified footnote b in Library Insert Fragmentation Time table
			  • Included figure showing Shortened Fragmentation Time Results

Part #	Revision	Date	Description of Change

15026495	B	February 2012	• *Low Throughput (LT)* protocol renamed *Low Sample (LS)* protocol
			• *High Throughput (HT)* protocol renamed *High Sample (HS)* protocol
			• Removed *TruSeq RNA Sample Prep v2* document catalog numbers
			• *Best Practices* section changes:
			  • Added *Handling Master Mix Reagents* section
			  • Renamed *AMPure XP Handling* to *Handling Magnetic Beads*
			  • Clarified *Usage Guidelines*
			  • Added *Equipment* section
			• *Tracking Tools* section changes:
			  • Removed sample sheet format guidelines and direct reader to sequencing analysis software user guide for detailed sample sheet guidelines
			  • *Illumina Experiment Manager* introduced
			• *Kit Contents* section changes:
			  • Kit part numbers added
			  • Bead Binding Buffer, RNA Purification Beads, and PCR Primer Cocktail part numbers changed
			• Removed instructions throughout protocol to "take care not to disturb the beads and change the tip after each sample", instead adding a note in the introduction section of each protocol to review the *Best Practices*

Part #	Revision	Date	Description of Change

15026495	B	February 2012 (continued)	• Removed steps in procedures to pre-heat thermal cycler lid and thermal cycler programs details, because thermal cycler programming instructions are duplicated in the preparation section of each procedure and now include lid pre-heating step
			• Changed "multiplexed" to "indexed" throughout documentation to refer to both dual-indexing and single-indexing
			• Purify and Fragment mRNA (LS protocol) - Revised procedure if starting protocol with 10–400 ng of previously isolated mRNA
			• Wash RBP - Remove the adhesive seal from plate ***before*** placing the plate on the magnet to avoid disturbing the beads
			• Indicated Second Strand Master Mix requires 1 tube per 48 reactions
			• Make CDP - Corrected SuperScript II to First Strand Master Mix tube ratio
			• Clean Up CDP renamed Purify CDP
			• Ligate Adapters (LS protocol) - Included final step to transfer supernatant from CAP to PCR plate
			• *Enrich DNA Fragments*:
			  • Removed "one tube each" following PMM and PPC specified in preparation steps, because 1 tube per 48 reactions is required
			  • *Make PCR* (LS protocol) - mixing step modified to adjust pipette to 50 µl
			• Removed all indicators that *Normalize and Pool Libraries* procedure/section is optional, because normalization is required. Pooling remains optional
			• Make PDP - Updated pool sample information to include 2–24 libraries
			• *Appendix A - Alternate Fragmentation Protocol*:
			  • Updated thermal cycler Elution 2 - Frag - Prime program temperature
			  • Added average library size information

15026495	A	August 2011	Initial Release

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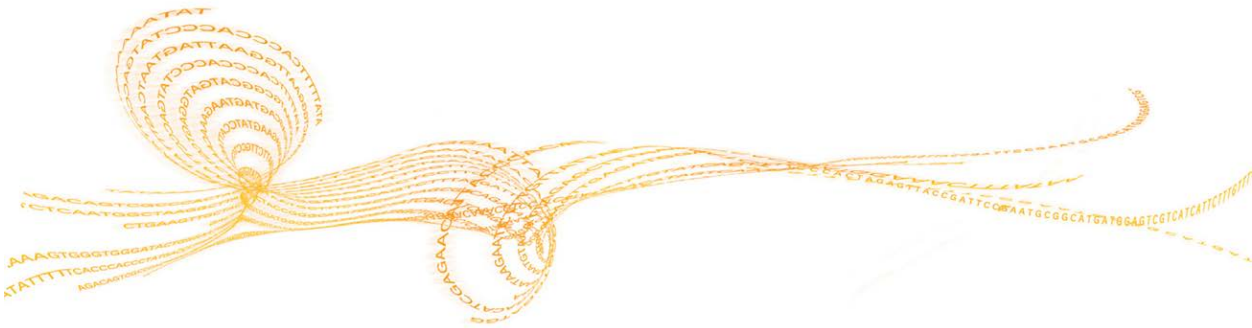
Overview

Chapter 1



Introduction

This protocol explains how to convert the mRNA in total RNA into a library of template molecules suitable for subsequent cluster generation and DNA sequencing using the reagents provided in the Illumina® TruSeq® RNA Sample Preparation Kit v2.

The first step in the workflow involves purifying the poly-A containing mRNA molecules using oligo-dT attached magnetic beads. Following purification, the mRNA 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. Second strand cDNA synthesis follows, using DNA Polymerase I and RNase H. The cDNA fragments then go through an end repair process, the addition of a single 'A' base, and then ligation of the adapters. The products are then purified and enriched with PCR to create the final cDNA library.

The sample preparation protocol offers:

Streamlined Workflow

- Master-mixed reagents reduce reagent containers, pipetting and hands-on time
- Universal adapter for preparation of mRNA samples

Higher Throughput

- 24 indexed adapters (12 per Set A kit and 12 per Set B kit) allow for simultaneous preparation of 96 indexed mRNA samples for sequencing when both kits are used
- Volumes optimized for standard 96-well plate

Improved Troubleshooting

- Process control checks built-in for QC

Universal Index Adapter Tags All Samples

- Additional adapters and primers not necessary

Protocol Features

This guide documents the sample preparation protocol using the Illumina TruSeq RNA Sample Prep Kit v2 or Alternate Kit.

- Chapter 2 Low Sample (LS) Protocol explains how to perform the TruSeq RNA Sample Preparation v2 using the Low Sample Protocol
- Chapter 3 High Sample (HS) Protocol explains how to perform the TruSeq RNA Sample Preparation v2 using the High Sample Protocol

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

Table 1 Protocol Features

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

RNA Input Recommendations

It is important to follow the TruSeq RNA Sample Preparation v2 input recommendations.

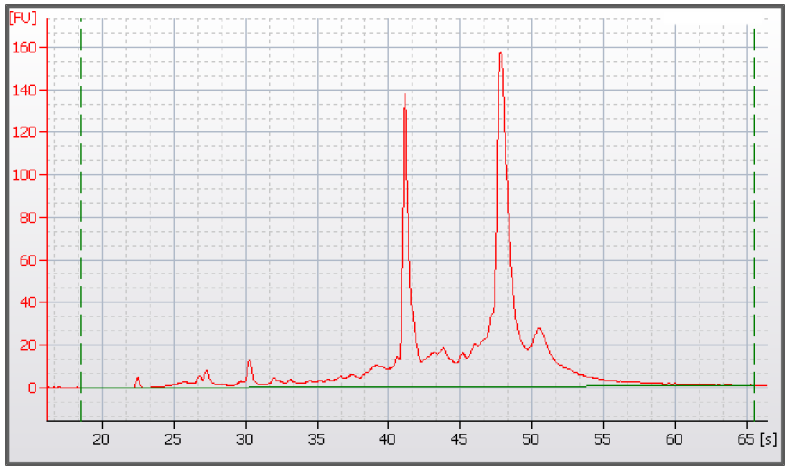
Total RNA Input

- Optimization
  - The TruSeq RNA Sample Prep v2 protocols are optimized for 0.1–1 µg of total RNA.
  - Lower amounts might result in inefficient ligation and low yield.
- Testing
  - The TruSeq RNA Sample Prep v2 protocols have been tested using 0.1–1 µg of high-quality universal human reference total RNA as input.
  - Use of RNA from other species, tissues, or qualities might require further optimization regarding the initial input amount.
- The protocol recommends diluting the in-line controls for tracking the steps involved in converting dsDNA into libraries.
  - The dilution is optimized for 0.1–1 µg of high-quality input RNA.
  - When using less RNA or RNA with very low mRNA content, these controls might need further dilution.
  - If no controls are added, use Resuspension Buffer in place of the controls in the protocol.
- It is important to know the quality of the RNA starting material. The fragmentation conditions were optimized for high-quality RNA.
  - Using the same fragmentation conditions for degraded RNAs, which are shorter than full length RNA, cause the libraries to be shorter. Shorter libraries can result in low yield or failure of the protocol.
  - Use of degraded RNA can result in low yield, over-representation of the 3' ends of the RNA molecules, or failure of the protocol.
  - Illumina recommends that you check total RNA integrity following isolation using an Agilent Technologies 2100 Bioanalyzer for human (or mammalian) samples with an RNA Integrity Number (RIN) value ≥ 8.
  - RNA that has DNA contamination results in an underestimation of the amount of RNA used.

- Illumina recommends including a DNase step with the RNA isolation method. However, contaminant DNA is removed during mRNA purification.

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

Figure 1 Starting RNA Bioanalyzer Trace



Alternatively, you can run a formaldehyde 1% agarose gel and judge the integrity of RNA upon staining with ethidium bromide.

- High-quality RNA shows a 28S rRNA band at 4.5 kb at twice the intensity of the 18S rRNA band at 1.9 kb.
- Both kb determinations are relative to an RNA 6000 ladder.
- The mRNA appears as a smear from 0.5–12 kb.

Purified mRNA Input

You can also use previously isolated mRNA as starting material. Use the entire fraction of mRNA purified from 0.1–1 µg of total RNA. If you start with isolated mRNA, follow the Illumina recommendations for isolated mRNA specified in the introduction of the Purify and Fragment mRNA procedures. Begin mRNA fragmentation with *Incubate RFP* on page 20 for LS processing or *Incubate RFP* on page 59 for HS processing.

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

NOTE

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

The control molecules work through the design of their ends. Controls are added to the reactions before their corresponding step in the protocol. Their end structures match the end structures of a DNA molecule that has not gone through the step. If the step is successful, the control molecule is modified to participate in downstream reactions of library generation and resulting in sequencing data. If the step fails, the control molecule does not go forward in the process and no sequencing data are generated. Using 1 µg of starting material, the controls yield approximately 0.2% of clusters, although the yield can vary based on library yield.

Table 2 In-Line Control Functions

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

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

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

Additional Resources

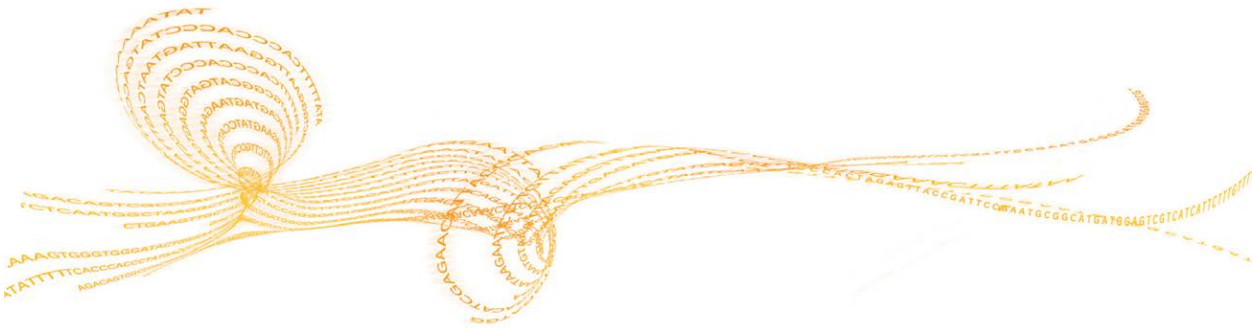
The following resources are available for TruSeq RNA Sample Preparation v2 protocol guidance and sample tracking. Access these and other resources on the Illumina website at support.illumina.com/sequencing/kits.ilmn. Then, select **TruSeq RNA Sample Prep Kit v2 Support**.

Resource	Description
Training	Illustrates elements of the TruSeq RNA Sample Preparation v2 process. Viewing these videos is recommended for new and less experienced users before starting sample preparation. Click **Training** on **TruSeq RNA Sample Prep Kit v2 Support**
Best Practices	Provides best practices specific to this protocol. Review these best practices before starting sample preparation. Topics include: • Handling Liquids • Handling Master Mix Reagents • Handling Magnetic Beads • Avoiding Cross-Contamination • Potential DNA Contaminants • Temperature Considerations • Equipment Click **Best Practices** on **TruSeq RNA Sample Prep Kit v2 Support**
TruSeq RNA Sample Preparation v2 Low Sample Experienced User Card and Lab Tracking Form (part # 15026498)	Provides LS protocol instructions, but with less detail than what is provided in this user guide. **New or less experienced users are advised to follow this user guide and not the EUC and LTF.** Click **Documentation & Literature** on **TruSeq RNA Sample Prep Kit v2 Support**

Resource	Description
TruSeq RNA Sample Preparation v2 High Sample Experienced User Card and Lab Tracking Form (part # 15026497)	Provides HS protocol instructions, but with less detail than what is provided in this user guide. **New or less experienced users are advised to follow this user guide and not the EUC and LTF.** Click **Documentation & Literature** on **TruSeq RNA Sample Prep Kit v2 Support**
Illumina Experiment Manager (IEM)	Enables you to create and edit appropriate sample sheets for Illumina sequencers and analysis software and record parameters for your sample plate. To download the software, click **Downloads** on **TruSeq RNA Sample Prep Kit v2 Support** To download the documentation, click **Documentation & Literature** on **TruSeq RNA Sample Prep Kit v2 Support**
BaseSpace®	Sequencing data analysis tool that also enables you to organize samples, libraries, pools, and sequencing runs in a single environment. For more information on BaseSpace see, support.illumina.com/sequencing/sequencing_software/basespace.ilmn
TruSeq Sample Preparation Pooling Guide (part # 15042173)	Provides TruSeq pooling guidelines for sample preparation. Review this guide before beginning library preparation. Click **Documentation & Literature** on **TruSeq RNA Sample Prep Kit v2 Support**
Sequencing Library qPCR Quantification Guide (part # 11322363)	Describes a qPCR method for quantifying sequencing by synthesis (SBS) libraries generated using the Illumina sample preparation protocols. Click **Documentation & Literature** on **TruSeq RNA Sample Prep Kit v2 Support**

Low Sample (LS) Protocol

Chapter 2



Introduction

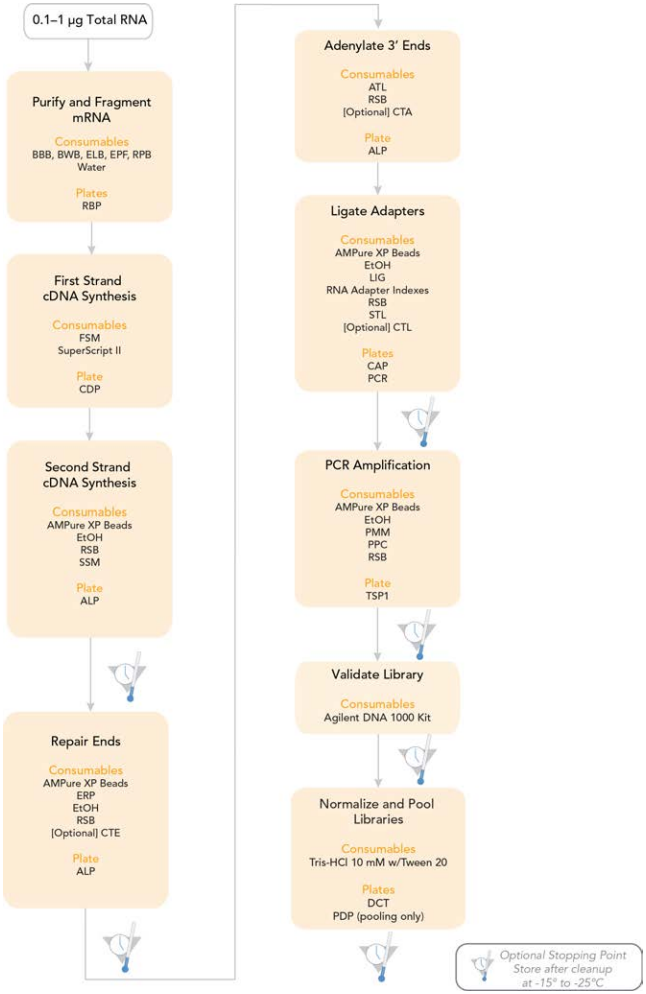
This chapter describes the TruSeq RNA Sample Preparation v2 LS protocol. Illumina recommends this protocol for processing 48 or fewer samples at one time. When processing more than 48 samples at one time, Illumina recommends following the Chapter 3 High Sample (HS) Protocol.

- Follow the protocol in the order described, using the specified volumes and incubation parameters.
- Before proceeding review the following:
  - Best Practices—See *Additional Resources* on page 9 for information on how to access TruSeq RNA Sample Preparation v2 Best Practices on the Illumina website.
  - Appendix A Supporting Information—Confirm your kit contents and make sure that you have obtained all of the requisite equipment and consumables for the LS protocol.

Sample Prep Workflow

The following illustrates the processes of the TruSeq RNA Sample Preparation v2 LS protocol to prepare templates using 24 indexed adapters.

Figure 2 TruSeq RNA Sample Preparation v2 LS Workflow



Prepare Adapter Setup

If you are pooling, use IEM or BaseSpace to record information about your samples before beginning library preparation.

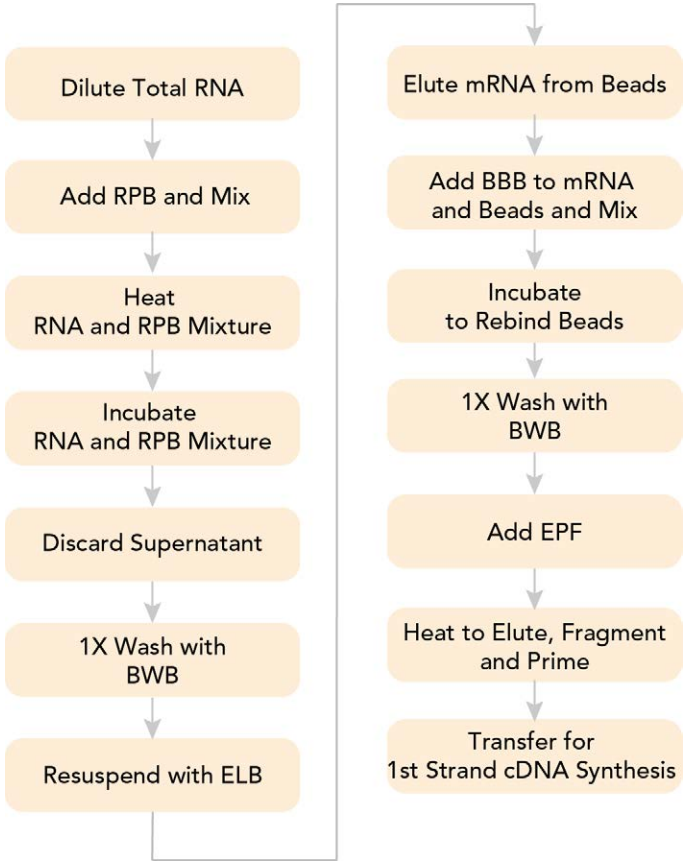
- Do one of the following:
  - Use IEM to create and edit sample sheets for Illumina sequencers and analysis software. See *Additional Resources* on page 9 for information on how to download IEM software and documentation from the Illumina website.
  - Use BaseSpace to organize samples, libraries, pools, and a run for Illumina sequencers and analysis software. See *Additional Resources* on page 9 for information on how to access BaseSpace or download BaseSpace documentation from the Illumina website.
- Review planning steps in the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 9 for information on how to download the guide from the Illumina website.

Illumina recommends arranging samples that will be combined into a common pool in the same row. Include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.

Purify and Fragment mRNA

This process purifies the polyA containing mRNA molecules using oligo-dT attached magnetic beads using two rounds of purification. During the second elution of the polyA RNA, the RNA is also fragmented and primed for cDNA synthesis. Reference the following diagram while performing the purification procedures:

Figure 3 TruSeq RNA Sample Preparation v2 Purification Workflow



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

NOTE
Allow the beads to fully pellet against the magnetic stand 5 minutes. Remove the supernatant from the beads immediately while the beads are still pelleted against the magnetic stand. Do not allow the pellets to dry.

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. Alternatively, you can start the protocol with 10–100 ng of previously isolated mRNA. If you do so, the mRNA must be concentrated into 5 µl or less before addition to the Elute, Prime, Fragment Mix. Concentrate by ethanol precipitation or on a QIAGEN MinElute column.

- If ethanol precipitation is used, resuspend the pellet in 18 µl Elute, Prime, Fragment Mix.
- If a QIAGEN MinElute column is used, elute the mRNA with 5 µl molecular biology-grade water and add 13 µl Elute, Prime, Fragment Mix. The use of the MinElute column results in a loss of up to 50% of the mRNA due to the low elution volume.

In either case, heat the mRNA in Elute, Prime, Fragment Mix to fragment at *Incubate RFP* on page 20 in this process.

Consumables

Item	Quantity	Storage	Supplied By
Bead Binding Buffer (BBB)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Bead Washing Buffer (BWB)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Elute, Prime, Fragment Mix (EPF)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Elution Buffer (ELB)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	-25°C to -15°C	Illumina
RNA Purification Beads (RPB)	1 tube per 48 reactions	2°C to 8°C	Illumina
RBP (RNA Bead Plate) barcode label	1 label per plate	15°C to 30°C	Illumina

Item	Quantity	Storage	Supplied By
96-well 0.3 ml PCR plate	1	15°C to 30°C	User
Microseal 'B' adhesive seals	3	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	6	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	6	15°C to 30°C	User

Preparation

- Remove the following from -25°C to -15°C storage and thaw them at room temperature:
  - Bead Binding Buffer
  - Bead Washing Buffer
  - Elution Buffer
  - Elute, Prime, Fragment Mix
  - Resuspension Buffer

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

  NOTE
  After use in this procedure, store the Bead Binding Buffer, Bead Washing Buffer, and Elution Buffer at 2°C to 8°C for subsequent experiments.

- Remove the RNA Purification Beads tube from 2°C to 8°C storage and let stand to bring to room temperature.

- Pre-program the thermal cycler with the following programs:
  - Choose the pre-heat lid option and set to 100°C
  - 65°C for 5 minutes, 4°C hold—save as **mRNA Denaturation**
  - 80°C for 2 minutes, 25°C hold—save as **mRNA Elution 1**
  - 94°C for 8 minutes, 4°C hold—save as **Elution 2 - Frag - Prime**

NOTE
For inserts larger than 120–200 bp with a median size of 150 bp, see Appendix B Alternate Fragmentation Protocols.

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

Make RBP

1. Dilute the total RNA with nuclease-free ultra pure water to a final volume of 50 µl in the new 96-well 0.3 ml PCR plate labeled with the RBP barcode.
2. Vortex the room temperature RNA Purification Beads tube vigorously to resuspend the oligo-dT beads.
3. Add 50 µl RNA Purification Beads to each well of the RBP plate to bind the polyA RNA to the oligo-dT beads. Gently pipette the entire volume up and down 6 times to mix thoroughly.
4. Seal the RBP plate with a Microseal 'B' adhesive seal.

Incubate 1 RBP

1. Place the sealed RBP plate on the pre-programmed thermal cycler. Close the lid and select **mRNA Denaturation** (65°C for 5 minutes, 4°C hold) to denature the RNA and facilitate binding of the polyA RNA to the beads.
2. Remove the RBP plate from the thermal cycler when it reaches 4°C.
3. Place the RBP plate on the bench and incubate at room temperature for 5 minutes to allow the RNA to bind to the beads.

Wash RBP

1. Remove the adhesive seal from the RBP plate.

2 Place the RBP plate on the magnetic stand at room temperature for 5 minutes to separate the polyA RNA bound beads from the solution.

3 Remove and discard all of the supernatant from each well of the RBP plate.

4 Remove the RBP plate from the magnetic stand.

5 Wash the beads by adding 200 µl Bead Washing Buffer in each well of the RBP plate to remove unbound RNA. Gently pipette the entire volume up and down 6 times to mix thoroughly.

6 Place the RBP plate on the magnetic stand at room temperature for 5 minutes.

7 Centrifuge the thawed Elution Buffer at 600 × g for 5 seconds.

8 Remove and discard all of the supernatant from each well of the RBP plate. The supernatant contains most of the ribosomal and other non-messenger RNA.

9 Remove the RBP plate from the magnetic stand.

10 Add 50 µl Elution Buffer in each well of the RBP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.

11 Seal the RBP plate with a Microseal 'B' adhesive seal.

12 Store the Elution Buffer tube at 4°C.

Incubate 2 RBP

1 Place the sealed RBP plate on the pre-programmed thermal cycler. Close the lid and select **mRNA Elution 1** (80°C for 2 minutes, 25°C hold) to elute the mRNA from the beads. Both the mRNA and any contaminant rRNA that have bound the beads non-specifically are released.

2 Remove the RBP plate from the thermal cycler when it reaches 25°C.

3 Place the RBP plate on the bench at room temperature.

4 Remove the adhesive seal from the RBP plate.

Make RFP

1 Centrifuge the thawed Bead Binding Buffer at 600 × g for 5 seconds.

2 Add 50 µl Bead Binding Buffer to each well of the RBP plate. This allows mRNA to specifically rebind the beads, while reducing the amount of rRNA that non-specifically binds. Gently pipette the entire volume up and down 6 times to mix thoroughly.

3 Incubate the RBP plate at room temperature for 5 minutes and store the Bead Binding Buffer tube at 2°C to 8°C.

4 Place the RBP plate on the magnetic stand at room temperature for 5 minutes.

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

6 Remove the RBP plate from the magnetic stand.

7 Wash the beads by adding 200 µl Bead Washing Buffer in each well of the RBP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.

8 Store the Bead Washing Buffer tube at 2°C to 8°C.

9 Place the RBP plate on the magnetic stand at room temperature for 5 minutes.

10 Remove and discard all of the supernatant from each well of the RBP plate. The supernatant contains residual rRNA and other contaminants that were released in the first elution and did not rebind the beads.

11 Remove the RBP plate from the magnetic stand.

12 Add 19.5 µl Elute, Prime, Fragment Mix to each well of the RBP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly. The Elute, Prime, Fragment Mix contains random hexamers for RT priming and serves as the first strand cDNA synthesis reaction buffer.

13 Seal the RBP plate with a Microseal 'B' adhesive seal.

14 Store the Elute, Prime, Fragment Mix tube at -25°C to -15°C.

Incubate RFP

1 Place the sealed RBP plate on the pre-programmed thermal cycler. Close the lid and select **Elution 2 - Frag - Prime** (94°C for 8 minutes, 4°C hold) to elute, fragment, and prime the RNA.

2 Remove the RBP plate from the thermal cycler when it reaches 4°C and centrifuge briefly.

3 Proceed immediately to *Synthesize First Strand cDNA* on page 21.

Synthesize First Strand cDNA

This process reverse transcribes the cleaved RNA fragments primed with random hexamers into first strand cDNA using reverse transcriptase and random primers.

Consumables

Item	Quantity	Storage	Supplied By
First Strand Master Mix (FSM)	1 tube	-25°C to -15°C	Illumina
CDP (cDNA Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR plate	1	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	1	15°C to 30°C	User
SuperScript II Reverse Transcriptase	1 tube	-25°C to -15°C	User

Preparation

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

NOTE

The First Strand Master Mix 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 Master Mix into smaller aliquots and store at -25°C to -15°C.

- Pre-program the thermal cycler with the following program and save as **1st Strand**:
  - Choose the pre-heat lid option and set to 100°C
  - 25°C for 10 minutes
  - 42°C for 50 minutes
  - 70°C for 15 minutes
  - Hold at 4°C
- Apply a CDP barcode label to a new 96-well 0.3 ml PCR plate.

Make CDP

1. Place the RBP plate on the magnetic stand at room temperature for 5 minutes. Do not remove the plate from the magnetic stand.
2. Remove the adhesive seal from the RBP plate.
3. Transfer 17 µl of the supernatant (fragmented and primed mRNA) from each well of the RBP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CDP barcode.
4. Centrifuge the thawed First Strand Master Mix tube at 600 × g for 5 seconds.
5. Add 50 µl SuperScript II to the First Strand Master Mix tube. If you are not using the entire contents of the First Strand Master Mix tube, add SuperScript II at a ratio of 1 µl SuperScript II for each 9 µl First Strand Master Mix. Mix gently, but thoroughly, and centrifuge briefly.
   Label the First Strand Master Mix tube to indicate that the SuperScript II has been added.
6. Add 8 µl First Strand Master Mix and SuperScript II mix to each well of the CDP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.
7. Seal the CDP plate with a Microseal 'B' adhesive seal and centrifuge briefly.
8. Return the First Strand Master Mix tube to -25°C to -15°C storage immediately after use.

Incubate 1 CDP

1. Place the sealed CDP plate on the pre-programmed thermal cycler. Close the lid, and then select and run the **1st Strand** program.
   a. Choose the pre-heat lid option and set to 100°C
   b. 25°C for 10 minutes
   c. 42°C for 50 minutes
   d. 70°C for 15 minutes
   e. Hold at 4°C
2. When the thermal cycler reaches 4°C, remove the CDP plate from the thermal cycler and proceed immediately to *Synthesize Second Strand cDNA* on page 24.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand to generate ds cDNA. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Second Strand Master Mix (SSM)	1 tube per 48 reactions	-25°C to -15°C	Illumina
IMP (Insert Modification Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR plate	1	15°C to 30°C	User
AMPure XP beads	90 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User
RNase/DNase-free reagent reservoir (if using multichannel pipettes)	4	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	4	15°C to 30°C	User

Preparation

- Remove the Second Strand Master Mix from -25°C to -15°C storage and thaw at room temperature.
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.

- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 9 for information on how to access TruSeq RNA Sample Preparation v2 Best Practices on the Illumina website.
- 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.
- Apply an IMP barcode label to a new 96-well 0.3 ml PCR plate.

Add SSM

1. Centrifuge the thawed Second Strand Master Mix at 600 × g for 5 seconds.
2. Remove the adhesive seal from the CDP plate.
3. Add 25 µl thawed Second Strand Master Mix to each well of the CDP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.
4. Seal the CDP plate with a Microseal 'B' adhesive seal.

Incubate 2 CDP

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

Purify CDP

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

5 Remove and discard 135 µl of the supernatant from each well of the CDP plate.

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

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

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

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

9 Let the CDP plate stand at room temperature for 15 minutes to dry, and then remove the plate from the magnetic stand.

10 Centrifuge the thawed, room temperature Resuspension Buffer at 600 × g for 5 seconds.

11 Add 52.5 µl Resuspension Buffer to each well of the CDP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

12 Incubate the CDP plate at room temperature for 2 minutes.

13 Place the CDP plate on the magnetic stand at room temperature for 5 minutes.

14 Transfer 50 µl supernatant (ds cDNA) from the CDP plate to the new 96-well 0.3 ml PCR plate labeled with the IMP barcode.

SAFE STOPPING POINT
If you do not plan to proceed immediately to *Perform End Repair* on page 27, you can safely stop the protocol here. If you are stopping, seal the IMP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to seven days.

Perform End Repair

This process converts the overhangs resulting from fragmentation into blunt ends using an End Repair Mix. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the polymerase activity fills in the 5' overhangs.

Consumables

Item	Quantity	Storage	Supplied By
End Repair Mix (ERP)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
[Optional] End Repair Control (CTE)	1 tube per 48 reactions	-25°C to -15°C	Illumina
ALP (Adapter Ligation Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR plate	1	15°C to 30°C	User
AMPure XP beads	160 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- Remove the following from -25°C to -15°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.

  - End Repair Mix
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 9 for information on how to access TruSeq RNA Sample Preparation v2 Best Practices on the Illumina website.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the IMP plate from -25°C to -15°C storage, if it was stored at the conclusion of *Purify CDP* on page 25 and let stand to thaw at room temperature.
  - Centrifuge the thawed IMP plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed IMP plate.
- Pre-heat the thermal cycler to 30°C.
- Choose the thermal cycler pre-heat lid option and set to 100°C
- Apply an ALP barcode label to a new 96-well 0.3 ml PCR plate.

Make IMP

1 Do one of the following:
   - If using the in-line control reagent:
     - Centrifuge the thawed End Repair Control tube at 600 × g for 5 seconds.
     - Dilute the End Repair Control to 1/100 in Resuspension Buffer (1 µl End Repair Control + 99 µl Resuspension Buffer) before use. Discard the diluted End Repair Control after use.
     - Add 10 µl diluted End Repair Control to each well of the IMP plate that contains 50 µl ds cDNA.
   - If not using the in-line control reagent, add 10 µl Resuspension Buffer to each well of the IMP plate that contains 50 µl ds cDNA.

2 Add 40 µl End Repair Mix to each well of the IMP plate containing the ds cDNA. Gently pipette the entire volume up and down 10 times to mix thoroughly.

3 Seal the IMP plate with a Microseal 'B' adhesive seal.

Incubate IMP

1 Place the sealed IMP plate on the pre-heated thermal cycler. Close the lid and incubate at 30°C for 30 minutes.

2 Remove the IMP plate from the thermal cycler.

Clean Up IMP

1 Remove the adhesive seal from the IMP plate.

2 Vortex the AMPure XP beads until they are well dispersed.

3 Add 160 µl well-mixed AMPure XP beads to each well of the IMP plate containing 100 µl End Repair Mix. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

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

6 Using a 200 µl single channel or multichannel pipette set to 127.5 µl, remove and discard 127.5 µl of supernatant from each well of the IMP plate.

7 Repeat step 6 one time.

NOTE
Leave the IMP plate on the magnetic stand while performing the following 80% EtOH wash steps (8–10).

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

9 Incubate the IMP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

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

11 Let the IMP plate stand at room temperature for 15 minutes to dry, and then remove the plate from the magnetic stand.

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

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

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

15 Transfer 15 µl of supernatant from each well of the IMP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the ALP plate barcode.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Adenylate 3' Ends* on page 31, you can safely stop the protocol here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

Item	Quantity	Storage	Supplied By
A-Tailing Mix (ATL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
[Optional] A-Tailing Control (CTA)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	3	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	3	15°C to 30°C	User

Preparation

- Prepare an ice bucket.

- Remove the following from -25°C to -15°C storage. Thaw them at room temperature and then place them on ice.
  - A-Tailing Control

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

  - A-Tailing Mix
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the ALP plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up IMP* on page 29.
  - Let it thaw at room temperature.
  - Centrifuge the thawed ALP plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the ALP plate.
- Pre-program the thermal cycler with the following program and save as **ATAIL70**:
  - Choose the pre-heat lid option and set to 100°C
  - 37°C for 30 minutes
  - 70°C for 5 minutes
  - Hold at 4°C

Add ATL

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

Incubate 1 ALP

1. Place the sealed ALP plate on the pre-programmed thermal cycler. Close the lid, then select and run the **ATAIL70** program.
   a. Choose the pre-heat lid option and set to 100°C
   b. 37°C for 30 minutes
   c. 70°C for 5 minutes
   d. Hold at 4°C
2. When the thermal cycler temperature is 4°C, remove the ALP plate from the thermal cycler, then proceed immediately to *Ligate Adapters* on page 34.

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
Ligation Mix (LIG)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
RNA Adapter Indexes (AR001–AR016, AR018–AR023, AR025, AR027)	1 tube of each index being used, per column of 8 reactions	-25°C to -15°C	Illumina
Stop Ligation Buffer (STL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
[Optional] Ligation Control (CTL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Barcode labels for: • CAP (Clean Up ALP Plate) • PCR (Polymerase Chain Reaction)	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR plates	2	15°C to 30°C	User
AMPure XP beads	92 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	800 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	3	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	4–28	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	4–28	15°C to 30°C	User

Preparation

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

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

  - RNA Adapter Index tubes for the indexes being used
  - Stop Ligation Buffer
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 9 for information on how to access TruSeq RNA Sample Preparation v2 Best Practices on the Illumina website.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-program the thermal cycler as follows:
  - Choose the thermal cycler pre-heat lid option and set to 100°C
  - 30°C for 10 minutes
- 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, Illumina recommends arranging samples that will be combined into a common pool in the same row. Include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.

Add LIG

1 Centrifuge the thawed RNA Adapter Index tubes, Ligation Control (if using Ligation Control), and Stop Ligation Buffer tubes at 600 × g for 5 seconds.

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

3 Remove the adhesive seal from the ALP plate.

4 Do one of the following:
- If using the in-line control reagent:
  - Dilute the Ligation Control to 1/100 in Resuspension Buffer (1 µl Ligation Control + 99 µl Resuspension Buffer) before use. Discard the diluted Ligation Control after use.
  - Add 2.5 µl diluted Ligation Control to each well of the ALP plate.
- If not using the in-line control reagent, add 2.5 µl Resuspension Buffer to each well of the ALP plate.

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

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

7 Add 2.5 µl thawed RNA Adapter Index to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

8 Seal the ALP plate with a Microseal 'B' adhesive seal.

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 Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation. Gently pipette the entire volume up and down 10 times to mix thoroughly.

Clean Up ALP

1 Vortex the AMPure XP beads for at least 1 minute or until they are well dispersed.

2 Add 42 µl mixed AMPure XP beads to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

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

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

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

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

7 Incubate the ALP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

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

9 With the ALP plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes.

10 Remove the ALP plate from the magnetic stand.

11 Add 52.5 µl Resuspension Buffer to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly or until the beads are fully resuspended.

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

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

14 Transfer 50 µl of supernatant from each well of the ALP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CAP barcode. Take care not to disturb the beads.

15 Vortex the AMPure XP beads until they are well dispersed.

16 Add 50 µl mixed AMPure XP beads to each well of the CAP plate for a second cleanup. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

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

19 Remove and discard 95 µl of supernatant from each well of the CAP plate. Take care not to disturb the beads.

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

20 With the CAP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Take care not to disturb the beads.

21 Incubate the CAP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

22 Repeat steps 20 and 21 one time for a total of two 80% EtOH washes.

23 With the CAP plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes, and then remove the plate from the magnetic stand.

24 Add 22.5 µl Resuspension Buffer to each well of the CAP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly or until the beads are fully resuspended.

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

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

27 Transfer 20 µl of supernatant from each well of the CAP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the PCR barcode. Take care not to disturb the beads.

SAFE STOPPING POINT
If you do not plan to proceed immediately to *Enrich DNA Fragments* on page 39, you can safely stop the protocol here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Enrich DNA Fragments

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

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

Consumables

Item	Quantity	Storage	Supplied By
PCR Master Mix (PMM)	1 tube per 48 reactions	-25°C to -15°C	Illumina
PCR Primer Cocktail (PPC)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
TSP1 (Target Sample Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
96-well 0.3 ml PCR plate	1	15°C to 30°C	User
AMPure XP beads	50 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	2	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User

Item	Quantity	Storage	Supplied By
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the PCR Master Mix and PCR Primer Cocktail from -25°C to -15°C storage. Thaw them at room temperature and then place them on ice.
- Centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 × g for 5 seconds.
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the AMPure XP beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 9 for information on how to access TruSeq RNA Sample Preparation v2 Best Practices on the Illumina website.
- Remove the PCR plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 36.
  - Let it thaw at room temperature.
  - Centrifuge the thawed PCR plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed PCR plate.

- Pre-program the thermal cycler with the following program and save as **PCR**:
  - Choose the pre-heat lid option and set to 100°C
  - 98°C for 30 seconds
  - 15 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 10°C
- Apply a TSP1 barcode label to a new 96-well 0.3 ml PCR plate.

Make PCR

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

Amp PCR

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

Clean Up PCR

1. Remove the adhesive seal from the PCR plate.
2. Vortex the AMPure XP Beads until they are well dispersed.

3 Add 50 µl mixed AMPure XP Beads to each well of the PCR plate containing 50 µl of PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

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

6 Remove and discard 95 µl of 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 (7–9).

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

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

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

10 With the PCR plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes and then remove the plate from the magnetic stand.

11 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.

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

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

14 Transfer 30 µl of 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 immediately to *Validate Library* on page 43, you can safely stop the protocol here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -25°C to -15°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

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

NOTE

See *Additional Resources* on page 9 for information on how to download the Illumina *Sequencing Library qPCR Quantification Guide (part # 11322363)* from the Illumina website.

Quality Control

1. Do one of the following:
   - Load 1 µl of resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA-specific chip such as the Agilent DNA 1000.
   - Dilute 1 µl of resuspended construct with 1 µl RSB and load on an Advanced Analytical Fragment Analyzer using Standard Sensitivity NGS Fragment Analysis Kit.
2. Check the size and purity of the sample. Check the size and purity of the sample. The final product should be a band at approximately 260 bp (for single-read libraries).

Figure 4 Example of TruSeq RNA Sample Preparation v2 Library Size Distribution

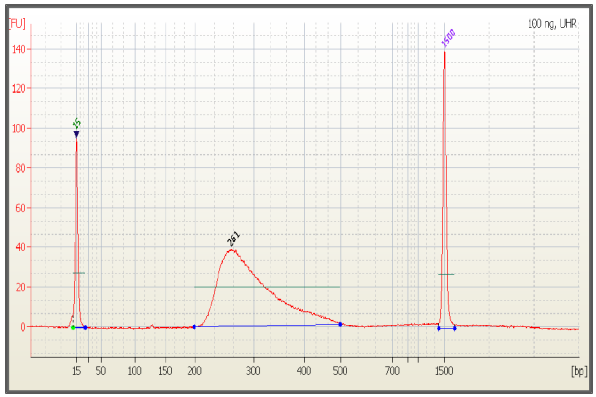
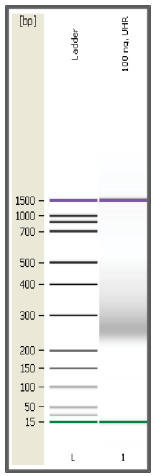


Figure 5 TruSeq RNA Sample Preparation v2 260 bp PCR Product



Normalize and Pool Libraries

This process describes how to prepare DNA templates for cluster generation. Indexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. Non-indexed DNA libraries are normalized to 10 nM in the DCT plate.

Consumables

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

Preparation

- Remove the TSP1 plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 41.
  - Let it thaw at room temperature.
  - Centrifuge the thawed TSP1 plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.
- Review the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See for information on how to download the guide from the Illumina website.
- Apply a DCT barcode label to a new 96-well MIDI plate.

- [For pooling only] Apply a PDP barcode label to a new 96-well 0.3 ml PCR plate.

Make DCT

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

   NOTE
   Depending on the yield quantification data of each sample library, the final volume in the DCT plate can vary from 10–400 µl.
3. Gently pipette the entire normalized sample library volume up and down 10 times to mix thoroughly.
4. Depending on the type of library you want to generate, do one of the following:
   - For non-pooled libraries, the protocol stops here. Do one of the following:
     - Proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina platform.
     - Seal the DCT plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C.
   - For pooled libraries, proceed to *Make PDP (for pooling only)*.

Make PDP (for pooling 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.
2. 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 is 10X the number of combined sample libraries and 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.

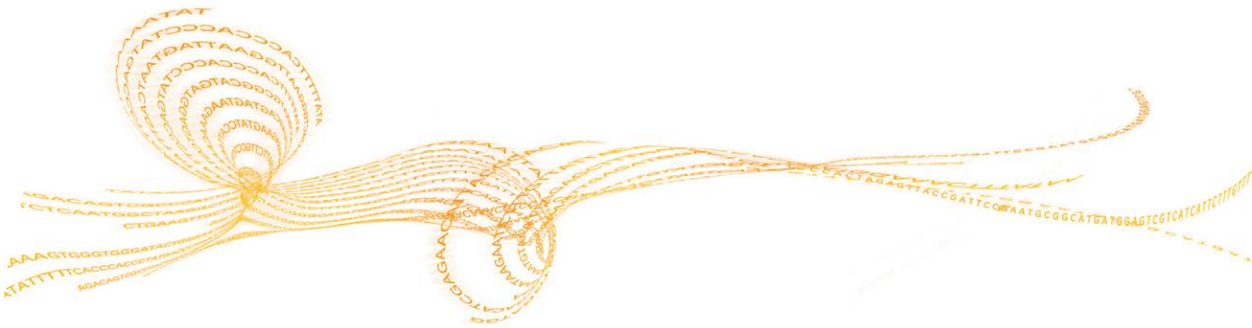
   NOTE
   Avoid pooling two samples with the same index.
3. Gently pipette the entire volume up and down 10 times to mix thoroughly.

4 Do one of the following:

- Proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina sequencing platform.
- Seal the PDP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C.

High Sample (HS) Protocol

Chapter 3



Introduction

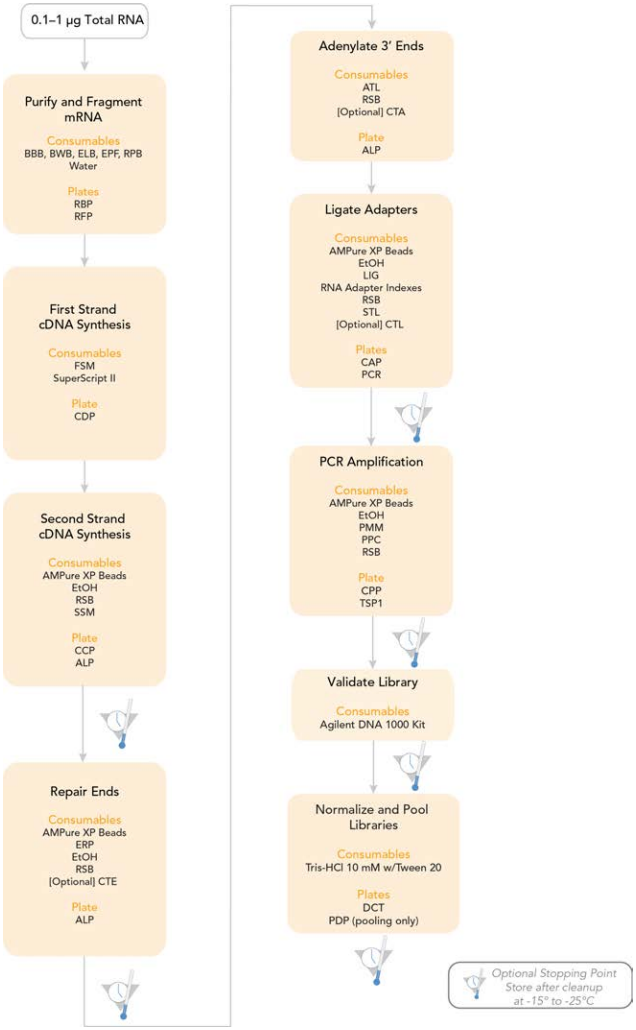
This chapter describes the TruSeq RNA Sample Preparation v2 HS protocol. Illumina recommends this protocol when processing more than 48 samples. When processing 48 or fewer samples at one time, Illumina recommends following the Chapter 2 Low Sample (LS) Protocol.

- Follow the protocol in the order described, using the specified volumes and incubation parameters.
- Before proceeding review the following:
  - Best Practices—See *Additional Resources* on page 9 for information on how to access TruSeq RNA Sample Preparation v2 Best Practices on the Illumina website.
  - Appendix A Supporting Information—To confirm your kit contents and make sure that you have obtained all of the requisite equipment and consumables for the HS protocol.

Sample Prep Workflow

The following illustrates the processes of the TruSeq RNA Sample Preparation v2 HS protocol to prepare templates using 24 indexed adapters.

Figure 6 TruSeq RNA Sample Preparation v2 HS Workflow



Prepare Adapter Setup

If you are pooling, use IEM or BaseSpace to record information about your samples before beginning library preparation.

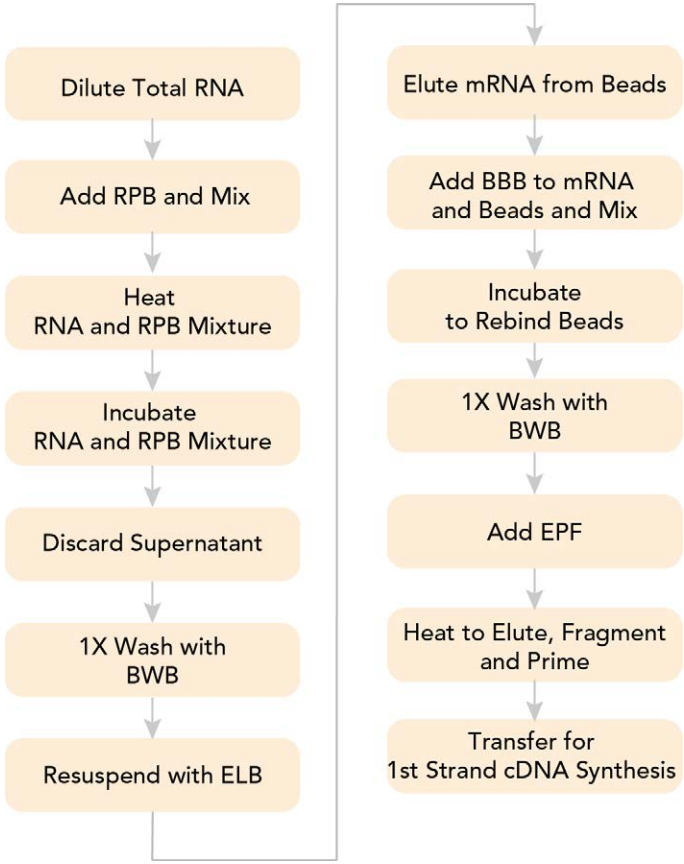
- Do one of the following:
  - Use IEM to create and edit sample sheets for Illumina sequencers and analysis software. See *Additional Resources* on page 9 for information on how to download IEM software and documentation from the Illumina website.
  - Use BaseSpace to organize samples, libraries, pools, and a run for Illumina sequencers and analysis software. See *Additional Resources* on page 9 for information on how to access BaseSpace or download BaseSpace documentation from the Illumina website.
- Review planning steps in the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 9 for information on how to download the guide from the Illumina website.

Illumina recommends arranging samples that will be combined into a common pool in the same row. Include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.

Purify and Fragment mRNA

This process purifies the polyA containing mRNA molecules using oligo-dT attached magnetic beads using two rounds of purification. During the second elution of the polyA RNA, the RNA is also fragmented and primed for cDNA synthesis. Reference the following diagram while performing the purification procedures:

Figure 7 TruSeq RNA Sample Preparation v2 Purification Workflow



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

NOTE
Allow the beads to fully pellet against the magnetic stand 5 minutes. Remove the supernatant from the beads immediately while the beads are still pelleted against the magnetic stand. Do not allow the pellets to dry.

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. Alternatively, you can start the protocol with 10–100 ng of previously isolated mRNA. If you do so, the mRNA must be concentrated into 5 µl or less before addition to the Elute, Prime, Fragment Mix. Concentrate by ethanol precipitation or on a QIAGEN MinElute column.

- If ethanol precipitation is used, resuspend the pellet in 18 µl Elute, Prime, Fragment Mix.
- If a QIAGEN MinElute column is used, elute the mRNA with 5 µl molecular biology-grade water and add 13 µl Elute, Prime, Fragment Mix. The use of the MinElute column results in loss of up to 50% of the mRNA due to the low elution volume.

In either case, heat the mRNA in Elute, Prime, Fragment Mix to fragment at *Incubate RFP* on page 20 in this process.

NOTE
For inserts larger than 120–200 bp with a median size of 150 bp, see Appendix B Alternate Fragmentation Protocols.

Consumables

Item	Quantity	Storage	Supplied By
Bead Binding Buffer (BBB)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Bead Washing Buffer (BWB)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Elute, Prime, Fragment Mix (EPF)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Elution Buffer (ELB)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	-25°C to -15°C	Illumina

Item	Quantity	Storage	Supplied By
RNA Purification Beads (RPB)	1 tube per 48 reactions	2°C to 8°C	Illumina
Barcode labels for: • RBP (RNA Bead Plate) • RFP (RNA Fragmentation Plate)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seals	7	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	6	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	6	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the following from -25°C to -15°C storage and thaw them at room temperature:
  - Bead Binding Buffer
  - Bead Washing Buffer
  - Elution Buffer
  - Elute, Prime, Fragment Mix
  - Resuspension Buffer

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

NOTE
After use in this procedure, store the Bead Binding Buffer, Bead Washing Buffer, and Elution Buffer at 2°C to 8°C for subsequent experiments.

- Remove the RNA Purification Beads tube from 2°C to 8°C storage and let stand to bring to room temperature.
- Pre-heat the microheating system to 65°C.
- Pre-program the thermal cycler with the following program and save as **Elution 2 - Frag - Prime**:
  - Choose the pre-heat lid option and set to 100°C
  - 94°C for 8 minutes
  - Hold at 4°C
- Make sure that the microplate shaker is properly calibrated to 1000 rpm using a stroboscope.
- Set the centrifuge to 15°C to 25°C, if refrigerated.
- Apply an RBP barcode label to a new 96-well MIDI plate.
- Apply an RFP barcode label to a new 96-well HSP plate.

Make RBP

1. Dilute the total RNA with nuclease-free ultra pure water to a final volume of 50 µl in the new 96-well MIDI plate labeled with the RBP barcode.
2. Vortex the room temperature RNA Purification Beads tube vigorously to resuspend the oligo-dT beads.
3. Add 50 µl RNA Purification Beads to each well of the RBP plate to bind the polyA RNA to the oligo-dT beads. Mix thoroughly as follows:
   a. Seal the RBP plate with a Microseal 'B' adhesive seal.
   b. Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.

Incubate 1 RBP

1. Place the sealed RBP plate on the pre-heated microheating system. Close the lid and incubate at 65°C for 5 minutes to denature the RNA and facilitate binding of the polyA RNA to the beads.
2. Remove the RBP plate from the microheating system and place on ice for 1 minute.
3. Place the RBP plate on the bench and incubate at room temperature for 5 minutes to allow the RNA to bind to the beads.
4. Pre-heat the microheating system to 80°C for the subsequent incubation.

Wash RBP

1. Remove the adhesive seal from the RBP plate.
2. Place the RBP plate on the magnetic stand at room temperature for 5 minutes to separate the polyA RNA bound beads from the solution.
3. Remove and discard all of the supernatant from each well of the RBP plate.
4. Remove the RBP plate from the magnetic stand.
5. Wash the beads by adding 200 µl Bead Washing Buffer in each well of the RBP plate to remove unbound RNA. Mix thoroughly as follows:
   a. Seal the RBP plate with a Microseal 'B' adhesive seal.
   b. Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.
6. Remove the adhesive seal from the RBP plate.
7. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
8. Centrifuge the thawed Elution Buffer at 600 × g for 5 seconds.
9. Remove and discard all of the supernatant from each well of the RBP plate. The supernatant contains most of the ribosomal and other non-messenger RNA.
10. Remove the RBP plate from the magnetic stand.
11. Add 50 µl Elution Buffer in each well of the RBP plate. Mix thoroughly as follows:
    a. Seal the RBP plate with a Microseal 'B' adhesive seal.
    b. Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.
12. Store the Elution Buffer tube at 4°C.

Incubate 2 RBP

1. Place the sealed RBP plate on the pre-heated microheating system. Close the lid and incubate at 80°C for 2 minutes to elute the mRNA from the beads. Both the mRNA and any contaminant rRNA that have bound the beads non-specifically are released.
2. Remove the RBP plate from the microheating system and place on ice for 1 minute.
3. Place the RBP plate on the bench at room temperature.
4. Remove the adhesive seal from the RBP plate.

Make RFP

1. Centrifuge the thawed Bead Binding Buffer at 600 × g for 5 seconds.
2. Add 50 µl Bead Binding Buffer to each well of the RBP plate. This allows mRNA to specifically rebind the beads, while reducing the amount of rRNA that non-specifically binds. Mix thoroughly as follows:
   a. Seal the RBP plate with a Microseal 'B' adhesive seal.
   b. Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.
3. Incubate the RBP plate at room temperature for 5 minutes and store the Bead Binding Buffer tube at 2°C to 8°C.
4. Remove the adhesive seal from the RBP plate.
5. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
6. Remove and discard all of the supernatant from each well of the RBP plate.
7. Remove the RBP plate from the magnetic stand.
8. Wash the beads by adding 200 µl Bead Washing Buffer in each well of the RBP plate. Mix thoroughly as follows:
   a. Seal the RBP plate with a Microseal 'B' adhesive seal.
   b. Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.
9. Store the Bead Washing Buffer tube at 2°C to 8°C.
10. Remove the adhesive seal from the RBP plate.
11. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
12. Remove and discard all of the supernatant from each well of the RBP plate. The supernatant contains residual rRNA and other contaminants that were released in the first elution and did not rebind the beads.
13. Remove the RBP plate from the magnetic stand.
14. Add 19.5 µl Elute, Prime, Fragment Mix to each well of the RBP plate. The Elute, Prime, Fragment Mix contains random hexamers for RT priming and serves as the first strand cDNA synthesis reaction buffer. Mix thoroughly as follows:
    a. Seal the RBP plate with a Microseal 'B' adhesive seal.
    b. Shake the RBP plate on a microplate shaker continuously at 1000 rpm for 1 minute.

15 Remove the adhesive seal from the RBP plate.

16 Transfer the entire contents from each well of the RBP plate to the corresponding well of the new HSP plate labeled with the RFP barcode.

17 Seal the RFP plate with a Microseal 'B' adhesive seal.

18 Store the Elute, Prime, Fragment Mix tube at -25°C to -15°C.

Incubate RFP

1 Place the sealed RFP plate on the pre-programmed thermal cycler. Close the lid and select **Elution 2 - Frag - Prime** (94°C for 8 minutes, 4°C hold) to elute, fragment, and prime the RNA.

2 Remove the RFP plate from the thermal cycler when it reaches 4°C and centrifuge briefly.

3 Proceed immediately to *Synthesize First Strand cDNA* on page 60.

Synthesize First Strand cDNA

This process reverse transcribes the cleaved RNA fragments primed with random hexamers into first strand cDNA using reverse transcriptase and random primers.

Consumables

Item	Quantity	Storage	Supplied By
First Strand Master Mix (FSM)	1 tube	-25°C to -15°C	Illumina
CDP (cDNA Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	1	15°C to 30°C	User
SuperScript II Reverse Transcriptase	1 tube	-25°C to -15°C	User

Preparation

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

NOTE

The First Strand Master Mix 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 Master Mix into smaller aliquots and store at -25°C to -15°C.

- Pre-program the thermal cycler with the following program and save as **1st Strand**:
  - Choose the pre-heat lid option and set to 100°C
  - 25°C for 10 minutes
  - 42°C for 50 minutes
  - 70°C for 15 minutes
  - Hold at 4°C
- Make sure that the microplate shaker is properly calibrated to 1000 rpm using a stroboscope.
- Apply a CDP barcode label to a new 96-well HSP plate.

Make CDP

1. Place the RFP plate on the magnetic stand at room temperature for 5 minutes. Do not remove the plate from the magnetic stand.
2. Remove the adhesive seal from the RFP plate.
3. Transfer 17 µl of the supernatant (fragmented and primed mRNA) from each well of the RFP plate to the corresponding well of the new HSP plate labeled with the CDP barcode.
4. Centrifuge the thawed First Strand Master Mix tube at 600 × g for 5 seconds.
5. Add 50 µl SuperScript II to the First Strand Master Mix tube. Mix gently, but thoroughly and centrifuge briefly. If you are not using the entire contents of the First Strand Master Mix tube, add SuperScript II at a ratio of 1 µl SuperScript II for each 9 µl First Strand Master Mix.
   Label the First Strand Master Mix tube to indicate that the SuperScript II has been added.
6. Add 8 µl First Strand Master Mix and SuperScript II mix to each well of the CDP plate. Mix thoroughly as follows:
   a. Seal the CDP plate with a Microseal 'B' adhesive seal.
   b. Shake the CDP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.
7. Return the First Strand Master Mix tube to -25°C to -15°C storage immediately after use.

Incubate 1 CDP

1. Place the sealed CDP plate on the pre-programmed thermal cycler. Close the lid, and then select and run the **1st Strand** program.
   a. Choose the pre-heat lid option and set to 100°C
   b. 25°C for 10 minutes
   c. 42°C for 50 minutes
   d. 70°C for 15 minutes
   e. Hold at 4°C
2. When the thermal cycler reaches 4°C, remove the CDP plate from the thermal cycler and proceed immediately to *Synthesize Second Strand cDNA* on page 63.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand to generate ds cDNA. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Second Strand Master Mix (SSM)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Barcode labels for: • CCP (cDNA Clean Up Plate) • IMP (Insert Modification Plate)	1 label per plate	15°C to 30°C	Illumina
96-well MIDI plates	2	15°C to 30°C	User
AMPure XP beads	90 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	4	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	4	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	4	15°C to 30°C	User

Preparation

- Remove the Second Strand Master Mix from -25°C to -15°C storage and thaw at room temperature.

- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 9 for information on how to access TruSeq RNA Sample Preparation v2 Best Practices on the Illumina website.
- 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.
- Apply a CCP barcode label to a new 96-well MIDI plate.
- Apply an IMP barcode label to a new 96-well MIDI plate.

Add SSM

1. Centrifuge the thawed Second Strand Master Mix at 600 × g for 5 seconds.
2. Remove the adhesive seal from the CDP plate.
3. Add 25 µl thawed Second Strand Master Mix to each well of the CDP plate. Mix thoroughly as follows:
   a. Seal the CDP plate with a Microseal 'B' adhesive seal.
   b. Shake the CDP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.

Incubate 2 CDP

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

Purify CDP

1. Vortex the AMPure XP beads until they are well dispersed.
2. Add 90 µl well-mixed AMPure XP beads to each well of the new MIDI plate labeled with the CCP barcode.

3 Transfer the entire contents from each well of the CDP plate to the corresponding well of the CCP plate containing AMPure XP beads. Mix thoroughly as follows:
   a Seal the CCP plate with a Microseal 'B' adhesive seal.
   b Shake the CCP plate on a microplate shaker at 1800 rpm for 2 minutes.

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

5 Centrifuge the CCP plate at 280 × g for 1 minute.

6 Remove the adhesive seal from the CCP plate.

7 Place the CCP plate on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells.

8 Remove and discard 135 µl of supernatant from each well of the CCP plate.

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

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

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

11 Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.

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

13 Centrifuge the thawed, room temperature Resuspension Buffer at 600 × g for 5 seconds.

14 Add 52.5 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 1800 rpm for 2 minutes.

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

16 Centrifuge the CCP plate to 280 × g for 1 minute.

17 Remove the adhesive seal from the CCP plate.

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

19 Transfer 50 µl supernatant (ds cDNA) from the CCP plate to the new MIDI plate labeled with the IMP barcode.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Perform End Repair* on page 67, you can safely stop the protocol here. If you are stopping, seal the IMP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Perform End Repair

This process converts the overhangs resulting from fragmentation into blunt ends using an End Repair Mix. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the polymerase activity fills in the 5' overhangs.

Consumables

Item	Quantity	Storage	Supplied By
End Repair Mix (ERP)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
[Optional] End Repair Control (CTE)	1 tube per 48 reactions	-25°C to -15°C	Illumina
ALP (Adapter Ligation Plate) barcode label	1 label per plate	15°C to 30°C	Illumina
96-well MIDI plate	1	15°C to 30°C	User
AMPure XP beads	160 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seals	4	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the following from -25°C to -15°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.

  - End Repair Mix
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 9 for information on how to access TruSeq RNA Sample Preparation v2 Best Practices on the Illumina website.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the IMP plate from -25°C to -15°C storage, if it was stored at the conclusion of *Purify CDP* on page 64 and let stand to thaw at room temperature.
  - Centrifuge the thawed IMP plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed IMP plate.
- Pre-heat the microheating system to 30°C.
- Calibrate the microplate shaker with a stroboscope and set it to 1800 rpm.
- Apply an ALP barcode label to a new 96-well MIDI plate.

Make IMP

1. Do one of the following:
   - If using the in-line control reagent:
     - Centrifuge the thawed End Repair Control tube at 600 × g for 5 seconds.
     - Dilute the End Repair Control to 1/100 in Resuspension Buffer (1 µl End Repair Control + 99 µl Resuspension Buffer) before use. Discard the diluted End Repair Control after use.
     - Add 10 µl diluted End Repair Control to each well of the IMP plate that contains 50 µl ds cDNA.
   - If not using the in-line control reagent, add 10 µl Resuspension Buffer to each well of the IMP plate that contains 50 µl ds cDNA.
2. Add 40 µl End Repair Mix to each well of the IMP plate containing the ds cDNA. Mix thoroughly as follows:
   a. Seal the IMP plate with a Microseal 'B' adhesive seal.
   b. Shake the IMP plate on a microplate shaker at 1800 rpm for 2 minutes.
3. Centrifuge the IMP plate at 280 × g for 1 minute.

Incubate IMP

1. Place the sealed IMP plate on the pre-heated microheating system. Close the lid and incubate at 30°C for 30 minutes.
2. Remove the IMP plate from the microheating system and place the plate on ice until you are ready for the next step.

Clean Up IMP

1. Remove the adhesive seal from the IMP plate.
2. Vortex the AMPure XP beads until they are well dispersed.
3. Add 160 µl well-mixed AMPure XP beads to each well of the IMP plate containing 100 µl End Repair Mix. Mix thoroughly as follows:
   a. Seal the IMP plate with a Microseal 'B' adhesive seal.
   b. Shake the IMP plate on a microplate shaker at 1800 rpm for 2 minutes.

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

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

6 Remove the adhesive seal from the IMP plate.

7 Using a 200 µl single channel or multichannel pipette set to 127.5 µl, remove and discard 127.5 µl of supernatant from each well of the IMP plate.

8 Repeat step 7 one time.

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

9 With the IMP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well with a sample without disturbing the beads.

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

11 Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.

12 Let the IMP plate stand at room temperature for 15 minutes to dry, then remove the plate from the magnetic stand.

13 Resuspend the dried pellet in each well with 17.5 µl Resuspension Buffer. Mix thoroughly as follows:
   a Seal the IMP plate with a Microseal 'B' adhesive seal.
   b Shake the IMP plate on a microplate shaker at 1800 rpm for 2 minutes.

14 Centrifuge the IMP plate at 280 × g for 1 minute.

15 Remove the adhesive seal from the IMP plate.

16 Incubate the IMP plate at room temperature for 2 minutes.

17 Place the IMP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.

18 Transfer 15 µl of supernatant from each well of the IMP plate to the corresponding well of the new MIDI plate labeled with the ALP barcode.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Adenylate 3′ Ends* on page 72, you can safely stop the protocol here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

Item	Quantity	Storage	Supplied By
A-Tailing Mix (ATL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
[Optional] A-Tailing Control (CTA)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	3	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	3	15°C to 30°C	User

Preparation

- Prepare an ice bucket.

- Remove the following from -25°C to -15°C storage. Thaw them at room temperature and then place them on ice.
  - A-Tailing Control

    NOTE
    The use of the A-Tailing Control is optional and it can be replaced with the same volume of Resuspension Buffer.
  - A-Tailing Mix
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the ALP plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up IMP* on page 69.
  - Let it thaw at room temperature.
  - Centrifuge the thawed ALP plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the ALP plate.
- Pre-heat two microheating systems: system 1 to 37°C and system 2 to 70°C.

Add ATL

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

Incubate 1 ALP

1. Place the sealed ALP plate on the pre-heated microheating system 1. Close the lid and incubate at 37°C for 30 minutes.
2. Immediately after the 37°C incubation, remove the ALP plate from system 1 and place the plate on the pre-heated microheating system 2. Close the lid and incubate at 70°C for 5 minutes.
3. Set the microheating system 1 to 30°C in preparation for *Ligate Adapters*.
4. Immediately remove the ALP plate from the microheating system 2 and place the plate on ice for 1 minute.
5. Proceed immediately to *Ligate Adapters* on page 75.

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
Ligation Mix (LIG)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
RNA Adapter Indexes (AR001–AR016, AR018–AR023, AR025, AR027)	1 tube of each index being used, per column of 8 reactions	-25°C to -15°C	Illumina
Stop Ligation Buffer (STL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
[Optional] Ligation Control (CTL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Barcode labels for: • CAP (Clean Up ALP Plate) • PCR (Polymerase Chain Reaction)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
AMPure XP beads	92 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	800 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	7	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	4–28	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	4–28	15°C to 30°C	User

Preparation

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

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

  - RNA Adapter Index tubes for the indexes being used
  - Stop Ligation Buffer
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 9 for information on how to access TruSeq RNA Sample Preparation v2 Best Practices on the Illumina website.
- 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 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, Illumina recommends arranging samples that will be combined into a common pool in the same row. Include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.

Add LIG

1. Centrifuge the thawed RNA Adapter Index tubes, Ligation Control (if using Ligation Control), and Stop Ligation Buffer tubes at 600 × g for 5 seconds.
2. Immediately before use, remove the Ligation Mix tube from -25°C to -15°C storage.
3. Remove the adhesive seal from the ALP plate.
4. Do one of the following:
   - If using the in-line control reagent:
     - Dilute the Ligation Control to 1/100 in Resuspension Buffer (1 µl Ligation Control + 99 µl Resuspension Buffer) before use. Discard the diluted Ligation Control after use.
     - Add 2.5 µl diluted Ligation Control to each well of the ALP plate.
   - If not using the in-line control reagent, add 2.5 µl Resuspension Buffer to each well of the ALP plate.
5. Add 2.5 µl Ligation Mix to each well of the ALP plate.
6. Return the Ligation Mix tube back to -25°C to -15°C storage immediately after use.
7. Add 2.5 µl thawed RNA Adapter Index to each well of the ALP plate.
   a. Seal the ALP plate with a Microseal 'B' adhesive seal.
   b. Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.
8. Centrifuge the ALP plate at 280 × g for 1 minute.

Incubate 2 ALP

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

Add STL

1. Remove the adhesive seal from the ALP plate.

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

Clean Up ALP

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

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

9 With the ALP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.
10 Incubate the ALP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.
11 Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.
12 With the ALP plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes.

13 Remove the ALP plate from the magnetic stand.

14 Add 52.5 µl Resuspension Buffer to each well of the ALP plate. Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

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

16 Centrifuge the ALP plate at 280 × g for 1 minute.

17 Remove the adhesive seal from the ALP plate.

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

19 Transfer 50 µl of supernatant from each well of the ALP plate to the corresponding well of the new MIDI plate labeled with the CAP barcode. Take care not to disturb the beads.

20 Vortex the AMPure XP beads until they are well dispersed.

21 Add 50 µl mixed AMPure XP beads to each well of the CAP plate for a second cleanup. Mix thoroughly as follows:
   a Seal the CAP plate with a Microseal 'B' adhesive seal.
   b Shake the CAP plate on a microplate shaker at 1800 rpm for 2 minutes.

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

23 Centrifuge the CAP plate at 280 × g for 1 minute.

24 Remove the adhesive seal from the CAP plate.

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

26 Remove and discard 95 µl of supernatant from each well of the CAP plate. Take care not to disturb the beads.

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

27 With the CAP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Take care not to disturb the beads.

28 Incubate the CAP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

29 Repeat steps 27 and 28 one time for a total of two 80% EtOH washes.

30 With the CAP plate on the magnetic stand, let the samples air-dry at room temperature for 15 minutes.

31 Remove the CAP plate from the magnetic stand.

32 Add 22.5 µl Resuspension Buffer to each well of the CAP plate. Mix thoroughly as follows:
   a Seal the CAP plate with a Microseal 'B' adhesive seal.
   b Shake the CAP plate on a microplate shaker at 1800 rpm for 2 minutes.

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

34 Centrifuge the CAP plate at 280 × g for 1 minute.

35 Remove the adhesive seal from the CAP plate.

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

37 Transfer 20 µl of supernatant from each well of the CAP plate to the corresponding well of the new HSP plate labeled with the PCR barcode. Take care not to disturb the beads.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Enrich DNA Fragments* on page 81, you can safely stop the protocol here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Enrich DNA Fragments

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

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

Consumables

Item	Quantity	Storage	Supplied By
PCR Master Mix (PMM)	1 tube per 48 reactions	-25°C to -15°C	Illumina
PCR Primer Cocktail (PPC)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Barcode labels for: • CPP (Clean Up PCR Plate) • TSP1 (Target Sample Plate)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
AMPure XP beads	50 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User

Item	Quantity	Storage	Supplied By
Microseal 'A' film	1	15°C to 30°C	User
Microseal 'B' adhesive seals	3	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the PCR Master Mix and PCR Primer Cocktail from -25°C to -15°C storage. Thaw them at room temperature and then place them on ice.
- Centrifuge the thawed PCR Master Mix and PCR Primer Cocktail at 600 × g for 5 seconds.
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the AMPure XP beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the PCR plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 78.
  - Let it thaw at room temperature.
  - Centrifuge the thawed PCR plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed PCR plate.

- Pre-program the thermal cycler with the following program and save as **PCR**:
  - Choose the pre-heat lid option and set to 100°C
  - 98°C for 30 seconds
  - 15 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 10°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 thawed PCR Primer Cocktail to each well of the PCR plate.
2. Add 25 µl thawed PCR Master Mix to each well of the PCR plate.
   a. Seal the PCR plate with a Microseal 'A' film.

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

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

Amp PCR

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

Clean Up PCR

1. Remove the adhesive seal from the PCR plate.
2. Vortex the AMPure XP Beads until they are well dispersed.
3. Add 50 µl mixed AMPure XP Beads to each well of the new MIDI plate labeled with the CPP barcode.
4. Transfer the entire contents from each well of the PCR plate to the corresponding well of the CPP plate containing 50 µl mixed AMPure XP Beads. Mix thoroughly as follows:
   a. Seal the CPP plate with a Microseal 'B' adhesive seal.
   b. Shake the CPP plate on a microplate shaker at 1800 rpm for 2 minutes.
5. Incubate the CPP plate at room temperature for 15 minutes.
6. Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid is clear.
7. Remove the adhesive seal from the CPP plate.
8. Remove and discard 95 µl of 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 (9–11).

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

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

11 Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.

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

13 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 1800 rpm for 2 minutes.

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

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

16 Remove the adhesive seal from the CPP plate.

17 Transfer 30 µl of 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 immediately to *Validate Library* on page 86, you can safely stop the protocol here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -25°C to -15°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

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

NOTE

See *Additional Resources* on page 9 for information on how to download the Illumina *Sequencing Library qPCR Quantification Guide (part # 11322363)* from the Illumina website.

Quality Control

1. Do one of the following:
   - Load 1 µl of resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA-specific chip such as the Agilent DNA 1000.
   - Dilute 1 µl of resuspended construct with 1 µl RSB and load on an Advanced Analytical Fragment Analyzer using Standard Sensitivity NGS Fragment Analysis Kit.
2. Check the size and purity of the sample. Check the size and purity of the sample. The final product should be a band at approximately 260 bp (for single-read libraries).

Figure 8 Example of TruSeq RNA Sample Preparation v2 Library Size Distribution

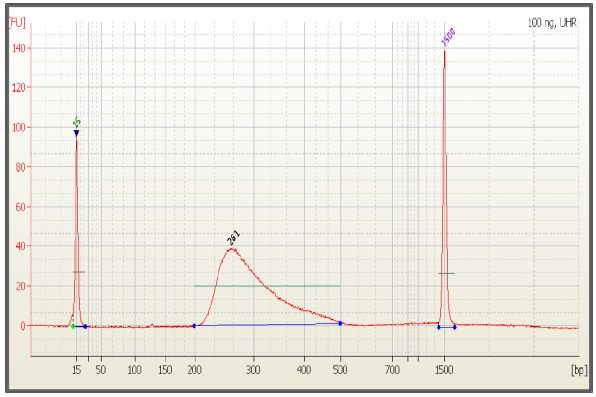
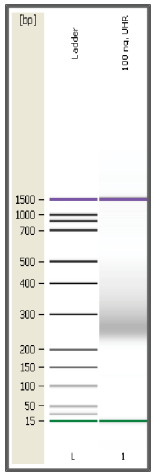


Figure 9 TruSeq RNA Sample Preparation v2 260 bp PCR Product



Normalize and Pool Libraries

This process describes how to prepare DNA templates for cluster generation. Indexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. Non-indexed DNA libraries are normalized to 10 nM in the DCT plate.

Consumables

Item	Quantity	Storage	Supplied By
Barcode labels for: • DCT (Diluted Cluster Template) • PDP (Pooled DCT Plate) (for pooling only)	1 label per plate	15°C to 30°C	Illumina
96-well HSP plate (for pooling only)	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
Microseal 'B' adhesive seals	4	15°C to 30°C	User
Tris-HCl 10 mM, pH8.5 with 0.1% Tween 20	Enough to normalize the concentration of each sample library to 10 nM	15°C to 30°C	User

Preparation

- Remove the TSP1 plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 84.
  - Let it thaw at room temperature.
  - Centrifuge the thawed TSP1 plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.
- Review the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See for information on how to download the guide from the Illumina website.
- Apply a DCT barcode label to a new 96-well MIDI plate.

- [For pooling only] Apply a PDP barcode label to a new 96-well HSP plate.

Make DCT

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

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

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

Make PDP (for pooling 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.

2 Transfer 10 µl of each normalized sample library to be pooled from the DCT plate to one well of the new HSP plate labeled with the PDP barcode.
The total volume in each well of the PDP plate is 10X the number of combined sample libraries and 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.

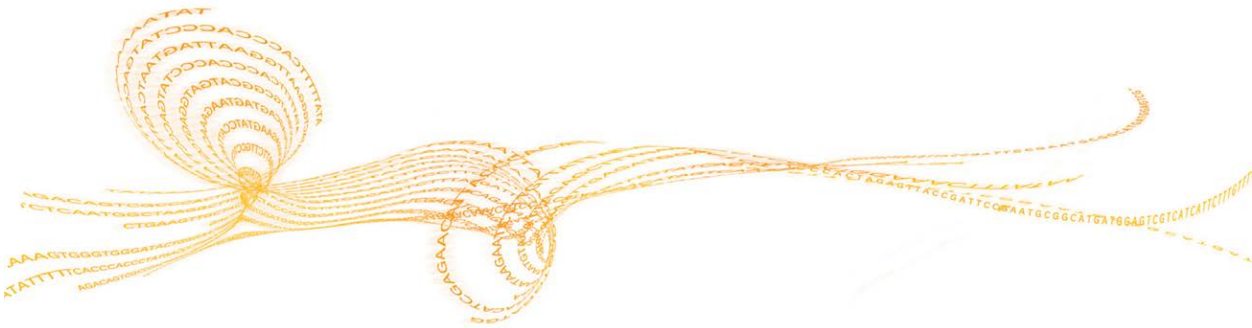
NOTE
Avoid pooling two samples with the same index.

3 Mix the PDP plate as follows:
   a Seal the PDP plate with a Microseal 'B' adhesive seal.
   b Shake the PDP plate on a microplate shaker at 1800 rpm for 2 minutes.

4 Do one of the following:
   - Proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina sequencing platform.
   - Seal the PDP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C.

Supporting Information

Appendix A



Introduction

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

Acronyms

Table 3 Acronyms

Acronym	Definition
ALP	Adapter Ligation Plate
ATL	A-Tailing Mix
BBB	Bead Binding Buffer
BWB	Bead Washing Buffer
CAP	Clean Up ALP Plate
CCP	cDNA Clean Up Plate
cDNA	Complimentary DNA
CDP	cDNA Plate
CPP	Clean Up PCR Plate
CTA	A-Tailing Control
CTE	End Repair Control
CTL	Ligation Control
DCT	Diluted Cluster Template
dsDNA	double-stranded DNA
ELB	Elution Buffer
EPF	Elute, Prime, Fragment Mix
ERP	End Repair Mix
EUC	Experienced User Card
FSM	First Strand Master Mix

Acronym	Definition
HSP	Hardshell Plate
HS	High Sample
HT	High Throughput
IMP	Insert Modification Plate
IEM	Illumina Experiment Manager
LIG	Ligation Mix
LS	Low Sample
LTF	Lab Tracking Form
PCR	Polymerase Chain Reaction
PDP	Pooled Dilution Plate
PMM	PCR Master Mix
PPC	PCR Primer Cocktail
RBP	RNA Bead Plate
RFP	RNA Fragmentation Plate
RPB	RNA Purification Beads
RSB	Resuspension Buffer
SAV	SequenceAnalysisViewer
SSM	Second Strand Master Mix
STL	Stop Ligation Buffer
TSP	Target Sample Plate

Kit Contents

Check to make sure that you have all of the reagents identified in this section before starting the TruSeq RNA Sample Preparation v2 protocol.

The TruSeq RNA Sample Prep Kit v2 is available in a Set A and a Set B. Each TruSeq RNA Sample Prep Kit v2 contains enough reagents to prepare up to 48 samples. When used together, sets A and B allow for pooling up to 24 samples using the 12 different indexes in each kit.

Table 4 TruSeq RNA Sample Prep v2 Kits

Kit Name	Catalog #	Number of Samples Supported	Number of Indexes
TruSeq RNA Sample Prep Kit v2 - Set A (48rxn)	RS-121-2001	48	12
TruSeq RNA Sample Prep Kit v2 - Set B (48rxn)	RS-121-2002	48	12

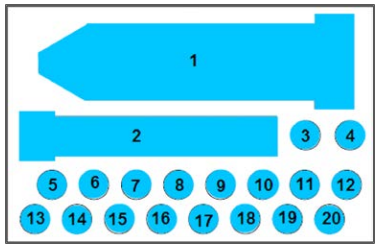
48 Samples, Boxes A and B

You receive either box A or B in the kit, depending on the set ordered. These boxes also contain plate barcode labels.

Store at -25°C to -15°C

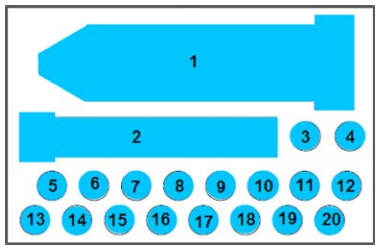
These boxes are shipped on dry ice. As soon as you receive them, store the following components at -25°C to -15°C.

Figure 10 TruSeq RNA Sample Prep Kit v2, Box A, part # 15025062



Slot	Reagent	Part #	Description
1	RSB	15026770	Resuspension Buffer
2	ERP	15012494	End Repair Mix
3	ATL	15012495	A-Tailing Mix
4	LIG	15026773	Ligation Mix
5	CTE	15026774	End Repair Control
6	CTA	15026775	A-Tailing Control
7	CTL	15026776	Ligation Control
8	STL	15012546	Stop Ligation Buffer
9	AR002	15026634	RNA Adapter Index 2
10	AR004	15026636	RNA Adapter Index 4
11	AR005	15026637	RNA Adapter Index 5
12	AR006	15026638	RNA Adapter Index 6
13	AR007	15026640	RNA Adapter Index 7
14	AR012	15026645	RNA Adapter Index 12
15	AR013	15024655	RNA Adapter Index 13
16	AR014	15024656	RNA Adapter Index 14
17	AR015	15024657	RNA Adapter Index 15
18	AR016	15024658	RNA Adapter Index 16
19	AR018	15024660	RNA Adapter Index 18
20	AR019	15024661	RNA Adapter Index 19

Figure 11 TruSeq RNA Sample Prep Kit v2, Box B, part # 15025063



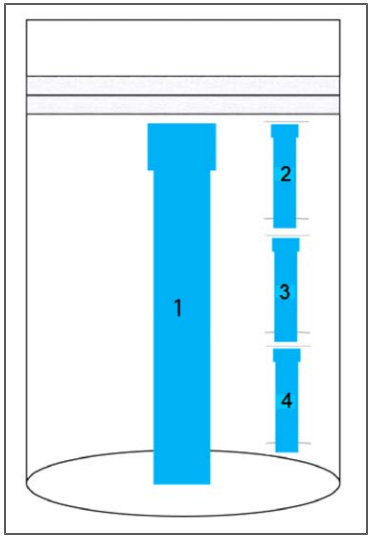
Slot	Reagent	Part #	Description
1	RSB	15026770	Resuspension Buffer
2	ERP	15012494	End Repair Mix
3	ATL	15012495	A-Tailing Mix
4	LIG	15026773	Ligation Mix
5	CTE	15026774	End Repair Control
6	CTA	15026775	A-Tailing Control
7	CTL	15026776	Ligation Control
8	STL	15012546	Stop Ligation Buffer
9	AR001	15026633	RNA Adapter Index 1
10	AR003	15026635	RNA Adapter Index 3
11	AR008	15026641	RNA Adapter Index 8
12	AR009	15026642	RNA Adapter Index 9
13	AR010	15026643	RNA Adapter Index 10
14	AR011	15026644	RNA Adapter Index 11
15	AR020	15024662	RNA Adapter Index 20
16	AR021	15024663	RNA Adapter Index 21
17	AR022	15024664	RNA Adapter Index 22
18	AR023	15024665	RNA Adapter Index 23
19	AR025	15024667	RNA Adapter Index 25
20	AR027	15024668	RNA Adapter Index 27

48 Samples - Box 1 of 2

Store as specified

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

Figure 12 TruSeq RNA Sample Prep Kit v2 48 Samples, (Box 1 of 2), part # 15027078



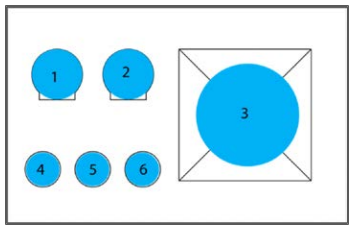
Slot	Reagent	Part #	Description	Storage Temperature
1	RPB	15026778	RNA Purification Beads	2°C to 8°C
2	DTE	15026766	CTE Dilution Tube	Room Temperature
3	DTA	15026805	CTA Dilution Tube	Room Temperature
4	DTL	15026807	CTL Dilution Tube	Room Temperature

48 Samples - Box 2 of 2

Store at -25°C to -15°C

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

Figure 13 TruSeq RNA Sample Prep Kit v2 Box 2, part # 15027387



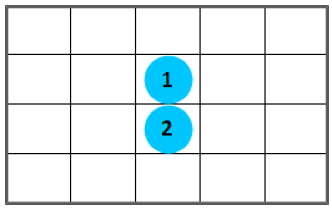
Slot	Reagent	Part #	Description
1	BBB	15026779	Bead Binding Buffer
2	ELB	15026780	Elution Buffer
3	BWB	15012925	Bead Washing Buffer
4	EPF	15026782	Elute, Prime, Fragment Mix
5	FSM	15026783	First Strand Master Mix
6	SSM	15026784	Second Strand Master Mix

48 Samples - PCR Box

Store at -25°C to -15°C

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

Figure 14 TruSeq RNA Sample Prep Kit v2, 48 Samples-PCR Box, part # 15027084



Slot	Reagent	Part #	Description
1	PMM	15026785	PCR Master Mix
2	PPC	15026786	PCR Primer Cocktail

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before starting the TruSeq RNA Sample Preparation v2 protocol. The requirement for some supplies is dependent upon the protocol performed (LS or HS) and these items are specified in separate tables.

NOTE
The TruSeq RNA Sample Preparation v2 protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Table 5 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

Consumable	Supplier
96-well 2 ml deep well plates (Optional - to aliquot reagents)	Thomson Instrument Company, part # 951652
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
Certified low-range ultra-agarose (Optional - to determine input RNA integrity)	Bio-Rad, part # 161-3107
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
MicroTube (6x16mm), AFA fiber with crimp-cap (Optional - for alternative fragmentation only)	Covaris, part # 520052
MinElute Gel Extraction Kit (Optional - if starting with previously isolated mRNA)	QIAGEN, part # 28604
Nuclease-free ultra pure water	General lab supplier
RNaseZap (to decontaminate surfaces)	General lab supplier
RNase/DNase-free eight-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
SuperScript II Reverse Transcriptase	Invitrogen, part # 18064-014
Tris-HCl 10 mM, pH8.5	General lab supplier
Tween 20	Sigma, part # P7949

Table 6 User-Supplied Consumables - Additional Items for LS Processing

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

Table 7 User-Supplied Consumables - Additional Items for HS Processing

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

Table 8 User-Supplied Equipment

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

Table 9 User-Supplied Equipment - Additional Items for HS Processing

Consumable	Supplier
High-Speed Microplate Shaker	VWR, catalog # • 13500-890 (110 V/120 V) or • 14216-214 (230 V)

Consumable	Supplier
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

One of the following:
Note: Two systems are recommended to support successive heating procedures.
• SciGene TruTemp Heating System
• Hybex Microsample Incubator
	• Illumina, catalog #
	  • SC-60-503 (115 V) or
	  • SC-60-504 (220 V)
	• SciGene, catalog #
	  • 1057-30-0 (115 V) or
	  • 1057-30-2 (230 V)

TruSeq RNA Sample Prep Kit v2 Indexed Adapter Sequences

The TruSeq RNA Sample Prep Kit v2 contains the following indexed adapter sequences.

NOTE

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

Table 10 TruSeq RNA Sample Prep Kit v2 Set A Indexed Adapter Sequences

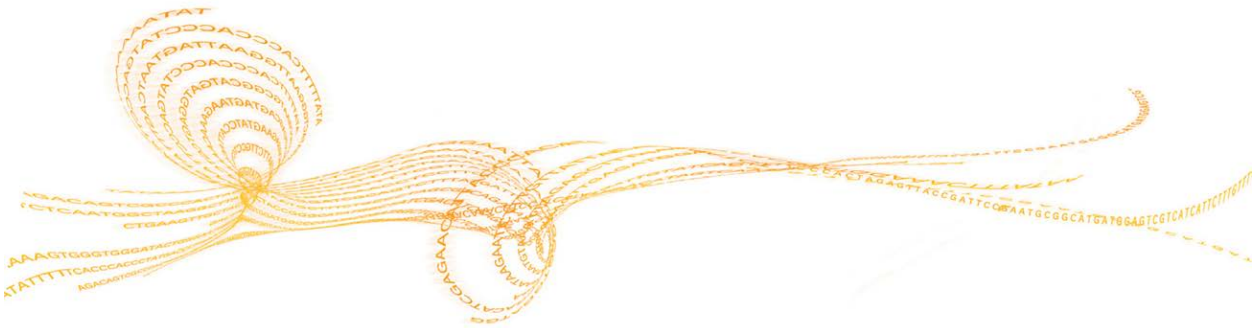
Adapter	Sequence	Adapter	Sequence
AR002	CGATGT(A)	AR013	AGTCAA(C)
AR004	TGACCA(A)	AR014	AGTTCC(G)
AR005	ACAGTG(A)	AR015	ATGTCA(G)
AR006	GCCAAT(A)	AR016	CCGTCC(C)
AR007	CAGATC(A)	AR018	GTCCGC(A)
AR012	CTTGTA(A)	AR019	GTGAAA(C)

Table 11 TruSeq RNA Sample Prep Kit v2 Set B Indexed Adapter Sequences

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

Alternate Fragmentation Protocols

Appendix B



Introduction

Fragmentation of the nucleic acids is required for optimal library preparation, clustering, and sequencing. The TruSeq RNA Sample Prep v2 fragmentation protocol for transcriptome analysis is performed on the RNA after mRNA purification using elevated temperatures. The fragmentation results in libraries with inserts ranging from 120–200 bp, with a median size of 150 bp. The TruSeq RNA Sample Prep v2 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. Two separate options are provided for varying the insert size of your library:

- Modify the fragmentation time
- Shear the sample after the synthesis of the ds cDNA.

Modify RNA Fragmentation Time

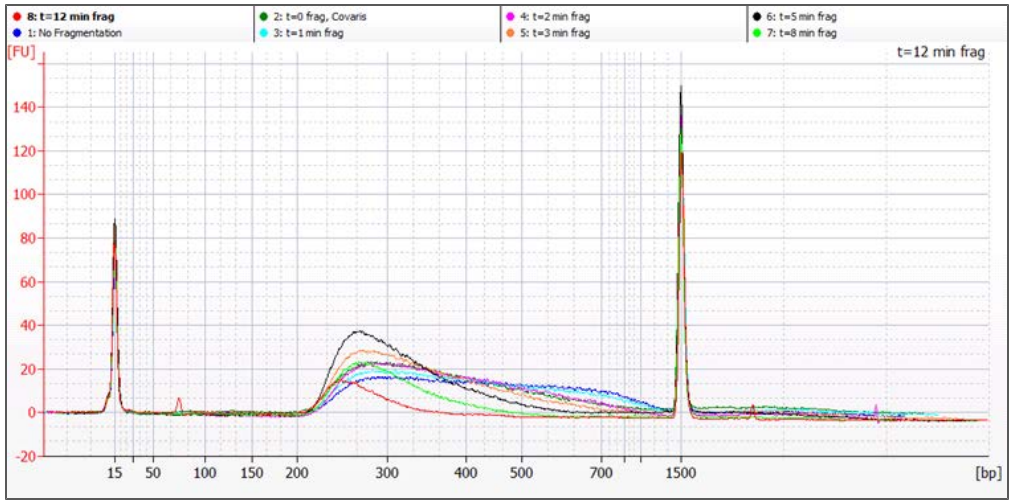
To modify the fragmentation of the RNA to allow for longer RNA fragments, the time of fragmentation can be shortened during the *Purify and Fragment mRNA* procedures. Modify the thermal cycler **Elution 2 - Frag - Prime** program: 94°C for X minutes followed by a 4°C hold for the thermal cycler. Determine X based on the length of the desired RNA. See Table 12 for a range of suggested times and sizes.

Table 12 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
Covaris[c]	130–280	180	385

a. Insert length determined after clustering and sequencing with a paired-end sequencing run.
b. Skip the Incubate RFP procedures (fragmentation) for samples requiring 0 minutes fragmentation time. Instead, place the sealed plate on the pre-heated thermal cycler. Close the lid and incubate the plate at 80°C for 2 minutes to elute the primed mRNA from the RNA Purification Beads. Then, immediately place the plate on the magnetic stand and proceed to the Synthesize First Strand cDNA process.
c. Covaris sheared sample was incubated for 2 minutes at 80°C instead of 94°C. See *Fragment Samples After ds cDNA Synthesis* on page 111.

Figure 15 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.

Fragment Samples After ds cDNA Synthesis

To shear the sample after the synthesis of the ds cDNA, during the Purify and Fragment mRNA procedures modify the thermal cycler **Elution 2 - Frag - Prime** program to 80°C for 2 minutes followed by a 4°C hold. Shearing during Purify and Fragment mRNA procedure elutes the mRNA and anneals the random primers without fragmenting the RNA. Proceed with the protocol through the Purify CDP procedures to purify the ds cDNA. The ds cDNA is in 50 µl of Resuspension Buffer. The cDNA can be transferred to a Covaris tube and sheared using a Covaris instrument as described in the following procedures.

User Supplied Consumables

- Covaris Tubes
- ds cDNA

Preparation

- Turn on the Covaris instrument at least 30 minutes before starting.
- Following the manufacturer's instructions, de-gas and pre-chill the water to a temperature of 3°C to 6°C. Start the fragmentation procedure at 6°C.
- Apply an IMP barcode label to a new 96-well plate.

Procedure

1 Shear each ds cDNA sample by adding 50 µl of each cDNA sample in a Covaris tube.

2 Fragment the ds cDNA using the following settings:

Option	Setting
Duty cycle	5%
Intensity	3
Bursts per second	200
Duration	80 seconds
Mode	Frequency sweeping
Power	33–34W
Temperature	5.5°C to 6°C

3 Seal the Covaris tube and centrifuge at 600 × g for 5 seconds.

4 Transfer the contents to the IMP plate.

5 Do one of the following:
- For LS processing, proceed to *Perform End Repair* on page 27
- For HS processing, proceed to *Perform End Repair* on page 67.

Index

M

P

Q

R

S

T

W

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 13 Illumina General Contact Information

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

Table 14 Illumina Customer Support Telephone Numbers

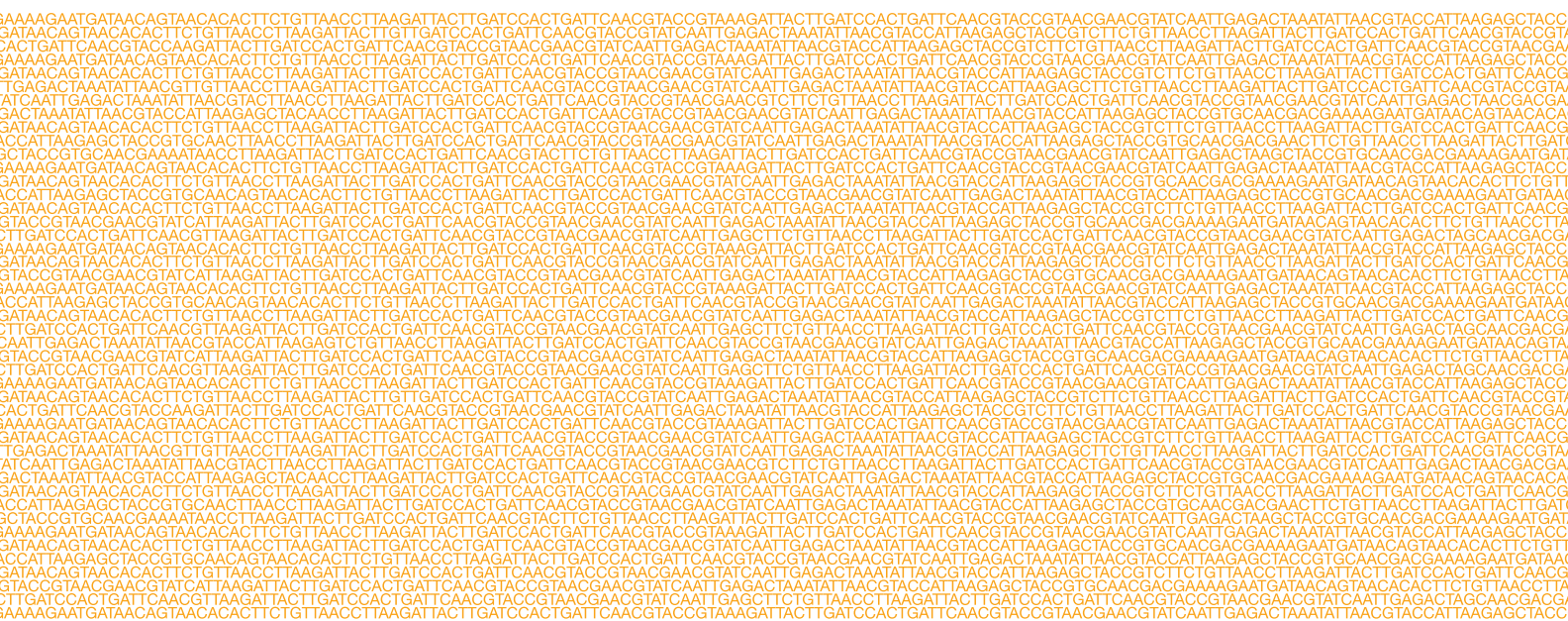
Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994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

Safety Data Sheets

Safety data sheets (SDSs) are available on the Illumina website at www.illumina.com/msds.

Product Documentation

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



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