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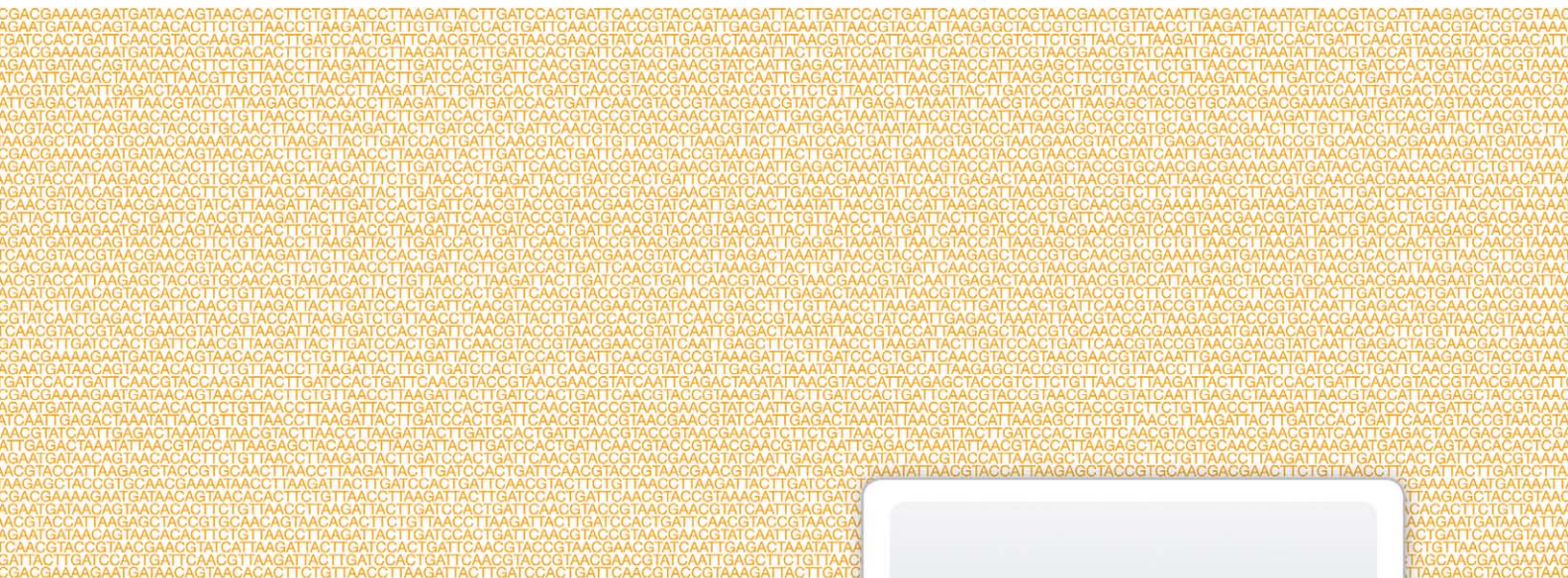
TruSeq™ RNA Sample Preparation v2 Guide



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Revision History

Part #	Revision	Date	Description of Change
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 v2 Kit.

The first step in the workflow involves purifying the poly-A containing mRNA molecules using poly-T oligo-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. This is followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. These 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 to reduce reagent containers, pipetting and hands-on time
- Universal adapter for preparation of mRNA samples

Higher Throughput

- Simultaneous preparation of 96 multiplexed mRNA samples
- 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

Audience and Purpose

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

- Chapter 3, explains how to perform the TruSeq RNA Sample Preparation v2 using the Low Throughput (LT) Protocol
- Chapter 4, explains how to perform the TruSeq RNA Sample Preparation v2 using the High Throughput (HT) Protocol

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

Table 1 Protocol Features

	Low Throughput	High Throughput
Number of Samples Processed	48 or fewer with indexed adapters	More than 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	Micro plate shaker

Getting Started

Chapter 2

Introduction

This chapter explains standard operating procedures and precautions for performing the TruSeq RNA Sample Preparation v2. You will also find lists of standard equipment and consumables.

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

Acronyms

Table 2 TruSeq RNA Sample Preparation v2 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
HT	High Throughput
IMP	Insert Modification Plate
ISP	Intermediate Source Plate
LIG	Ligation Mix
LT	Low Throughput
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	FNA Fragmentation Plate
RPB	RNA Purification Beads
RSB	Resuspension Buffer
STL	Stop Ligation Buffer
TSP	Target Sample Plate

Best Practices

When preparing mRNA libraries for sequencing, you should always adhere to good molecular biology practices.

RNA Handling

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

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

Liquid Handling

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

- Small differences in volumes (±0.5 µl) can sometimes give rise to very large differences in cluster numbers (~100,000).
- Small volume pipetting can be a source of potential error in protocols that require generation of standard curves, such as PicoGreen assays or qPCR, or those that require small but precise volumes, such as the Agilent Bioanalyzer.

- If small volumes are unavoidable, then due diligence should be taken to ensure that pipettes are correctly calibrated.
- Ensure that pipettes are not used at the volume extremes of their performance specifications.
- Care should be taken with solutions of high molecular weight double-stranded DNA (dsDNA). These can be viscous and not evenly dispersed, resulting in aliquot measurements that are not representative of the true concentration of the solution.
- To minimize pipetting errors, especially with small volume enzyme additions, prepare the reagents for multiple samples simultaneously. As a result, pipette once from the reagent tubes with a larger volume, rather than many times with 1 µl volumes. This will allow you to aliquot in a single pipetting movement to individual samples and standardize across multiple samples.

AMPure XP Handling

Following appropriate handling methods when working with Agencourt AMPure XP Beads:

NOTE
Cleanup procedures have only been verified using a 300 µl 96-well PCR or MIDI plate. Comparable performance is not guaranteed when using a microcentrifuge tube or other formats..

NOTE
Cleanup procedures have only been tested and validated using the magnetic stand specified in *Consumables and Equipment* on page 33 . Comparable performance is not guaranteed when using other magnets.

- Prior to use, allow the beads to come to room temperature.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous.
- **When performing the LT protocol**, after adding the beads to the reaction, mix the solution gently and thoroughly by pipetting up and down 10 times, making sure the liquid comes in contact with the beads and that the beads are resuspended homogeneously..
- **When performing the HT protocol**, after adding the beads to the reaction, seal the plate and shake the plate on a microplate shaker at 1,800 rpm for 2 minutes. Repeat, if necessary, until the color of the mixture appears homogeneous after mixing.

- Take care to minimize bead loss which can impact final yields.
- Change the tips for each sample.
- Let the mixed samples incubate for 15 minutes at room temperature for maximum recovery.
- When aspirating the cleared solution from the reaction plate and wash step, it is important to keep the plate on the magnetic stand and to not disturb the separated magnetic beads. Aspirate slowly to prevent the beads from sliding down the sides of the wells and into the pipette tips.
- To prevent the carryover of beads after elution, approximately 2.5 µl of supernatant are left when the eluates are removed from the bead pellet.
- For the wash steps, prepare fresh 80% ethanol. Ethanol tends to absorb water from the air, therefore, fresh 80% ethanol should be prepared for optimal results.
- Be sure to remove all of the ethanol from the bottom of the wells, as it may contain residual contaminants.
- Keep the reaction plate on the magnetic stand and let it air-dry at room temperature to prevent potential bead loss due to electrostatic forces. Allow for the complete evaporation of residual ethanol, as the presence of ethanol will impact the performance of the subsequent reactions. Illumina recommends at least 15 minutes drying time, but a longer drying time may be required.
- Use the Resuspension Buffer (RSB) for DNA elution.
- Resuspend the dried pellets using a single channel or multichannel pipette.
- When removing and discarding supernatant from the wells, use a single channel or multichannel pipette and take care not to disturb the beads.
- To maximize DNA recovery during elution, incubate the DNA/bead mix for 2 minutes at room temperature before placing the samples onto the magnet.

Avoid Cross-Contamination

Practice the following to avoid cross-contaimination:

- Open only one adapter at the time.
- Pipette carefully to avoid spillage.
- Clean pipettes and change gloves between handling different adapter stocks.
- Clean work surfaces thoroughly before and after the procedure.

Usage Guidelines

Illumina recommends these usage guidelines as the most efficient lab setup and pipetting process when performing the procedures specified in Chapter 3 Low-Throughput (LT) Protocol and Chapter 4 High-Throughput (HT) Protocol.

NOTE
The TruSeq RNA Sample Prep Kit v2 contains enough of each reagent to prepare 48 samples. If an alternate procedure is used, Illumina cannot guarantee that there will be enough of every reagent for 48 samples.

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

Preparing 24 or More Samples

When preparing 24 or more samples, follow these reagent setup and distribution guidelines as you perform each procedure described in Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol. Use a multichannel pipette with eight tips to perform all transfers from the reagent vessel to the sample plate.

Sample Distribution

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

- LT protocol - 0.3 ml PCR plate
- HT protocol - 96-well MIDI plate and 96-well HSP plate

Reagents in Reservoirs

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

- 80% Ethanol
- AMPure XP Beads
- Bead Binding Buffer
- Bead Washing Buffer
- Elution Buffer

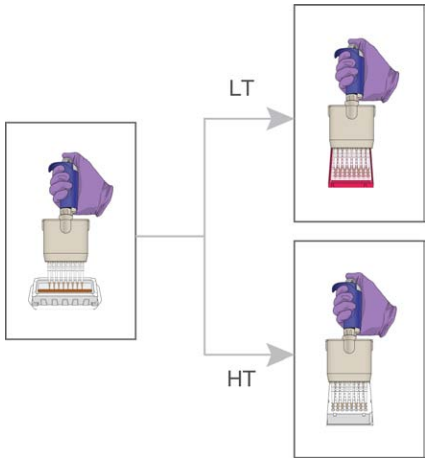
- Resuspension Buffer
- RNA Purification Beads

1. Determine the volume needed for each of the above reagents using the equation (# of samples x volume per sample) + 600 µl dead volume. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol for the required reagent volume per sample.
2. Fill a separate multichannel reagent reservoir with the determined amount of each reagent.

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

1. Using an eight tip multichannel pipette, transfer the reagent in the reservoir to the samples in the plate as follows, holding the pipette vertically. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol for the required reagent volume per sample.
   a. Pipette the required reagent volume per sample from the reservoir.
   b. Add the reagent to column 1 of the sample plate. Change the tips.
   c. Pipette the required reagent volume per sample from the reservoir.
   d. Add the reagent to column 2 of the sample plate. Change the tips.
   e. Repeat as needed for each column containing a sample.

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



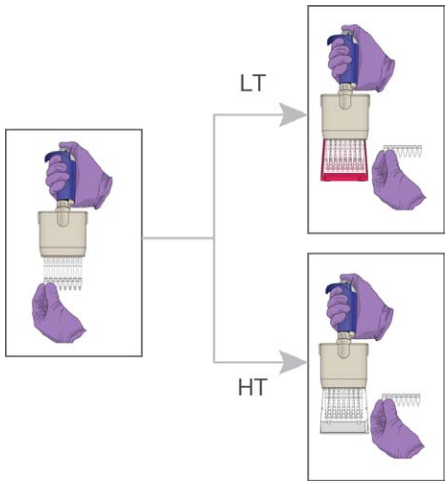
Reagents in Strip Tubes

When the remaining reagents not mentioned above, except the adapters, are required in the protocol, distribute each evenly across three wells of an 8-well strip tube. Add an allowance of 5 µl for dead volume per well.

When each reagent in an 8-well strip tube is required in the protocol, distribute each to the sample plate as follows:

1 Using an eight tip multichannel pipette, transfer the reagent in the 8-well strip tube to the samples in the plate as follows, holding the pipette vertically. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol for the required reagent volume per sample.
   a Pipette the reagent from the eight strip wells.
   b Add the reagent to column 1 of the sample plate. Change the tips.
   c Pipette the reagent from the eight strip wells.
   d Add the reagent to column 2 of the sample plate. Change the tips.
   e Repeat as needed for each column containing a sample.

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



Index Adapter Usage

When the index adapters are required in the protocol, do one of the following:

- Add 2.5 µl of the appropriate/desired adapter index individually to each well of the plate containing a sample, using a single channel pipette.
- Using an 8-well strip tube:
  - Distribute the index adapters into the wells of an 8-well strip tube, with a different adapter in each well.
  - Add 2.5 µl of the appropriate/desired adapter index from the well of the 8-well strip tube to each well of the plate containing a sample, using a multichannel pipette.

Preparing 12–24 Samples

When preparing 12–24 samples, follow these reagent setup and distribution guidelines as you perform each procedure described in Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol. Use a multichannel pipette with three tips to perform all transfers from the reagent vessel to the sample plate.

Sample Distribution

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

- LT protocol - 0.3 ml PCR plate
- HT protocol - 96-well MIDI plate and 96-well HSP plate

Reagents in Reservoirs

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

- 80% Ethanol
- AMPure XP Beads
- Bead Washing Buffer
- Resuspension Buffer

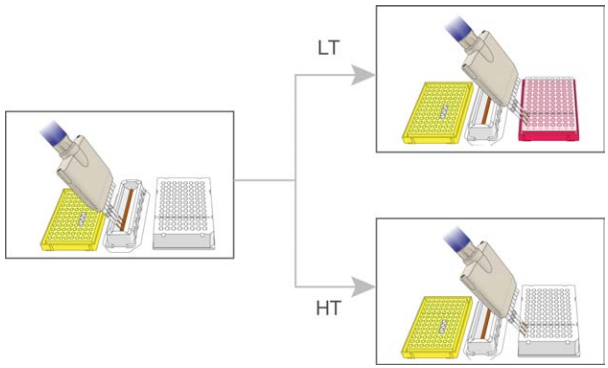
1. Determine the volume needed using the equation (# of samples x volume per sample) + 600 µl dead volume. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol for the required reagent volume per sample.

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

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

1 Using an multichannel pipette with three tips, transfer the reagent in the reservoir to the samples in the plate as follows, holding the pipette vertically. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol for the required reagent volume per sample.
   a Pipette the required reagent volume per sample from the reservoir.
   b Add the reagent to row 1 of the sample plate. Change the tips.
   c Pipette the required reagent volume per sample from the reservoir.
   d Add the reagent to row 2 of the sample plate. Change the tips.
   e Repeat as needed for each row containing a sample.

Figure 3 Transfer Reagent from Reservoir to Sample Plate with 12–24 Samples



Reagents in Deep Well Plates

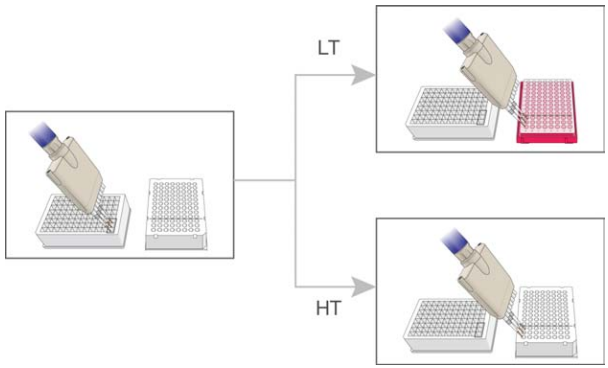
When each of the following reagents are required in the protocol, distribute each into a separate well, in the same row of a deep well plate, adding 75 µl dead volume.

- RNA Purification Beads
- Elution Buffer
- Bead Binding Buffer

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

1 Using an multichannel pipette with three tips, transfer the reagent in the deep well plate to the samples in the plate as follows, holding the pipette vertically. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol for the required reagent volume per sample.
   a Pipette the reagent from the deep well plate.
   b Add the reagent to row 1 of the sample plate. Change the tips.
   c Pipette the reagent from the deep well plate.
   d Add the reagent to row 2 of the sample plate. Change the tips.
   e Repeat as needed for each row containing a sample.

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



Reagents in Strip Tubes

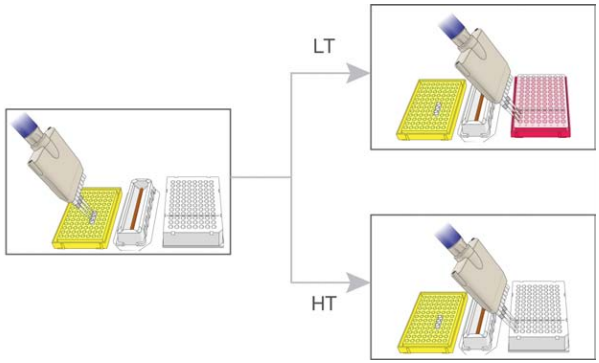
When the remaining reagents not mentioned above, except the adapters, are required in the protocol, distribute each evenly across the three wells of an 8-well strip tube. Add an allowance of 5 µl for dead volume per well.

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

1 Using an multichannel pipette with three tips, transfer the reagent in the strip tube to the samples in the plate as follows, holding the pipette vertically. Reference Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol for the required reagent volume per sample.
   a Pipette the reagent from the three strip wells.
   b Add the reagent to row 1 of the sample plate. Change the tips.

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

Figure 5 Transfer Reagent from Strip Tube to Sample Plate with 12–24 Samples



Index Adapter Usage

Add 2.5 µl of the appropriate/desired adapter index individually to each well of the plate containing a sample, using a single channel pipette.

Preparing Less Than 12 Samples

When preparing less than 12 samples, follow these reagent setup and distribution guidelines as you perform each procedure described in Chapter 3 Low-Throughput (LT) Protocol or Chapter 4 High-Throughput (HT) Protocol.

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

Temperature Considerations

Temperature is an important consideration for making cDNA libraries.

- Keep libraries at temperatures ≤37°C.
- Avoid elevated temperatures, particularly in the steps preceding the adapter ligation.

- mRNA fragments that have a high AT content are more likely to denature into single strands than GC-rich fragments, which can result in an increased probability of creating a bias in the sequencing coverage.
- Temperature is less of an issue after the adapters have been ligated onto the ends of the ds cDNA.

RNA Input Recommendations

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

Total RNA Input

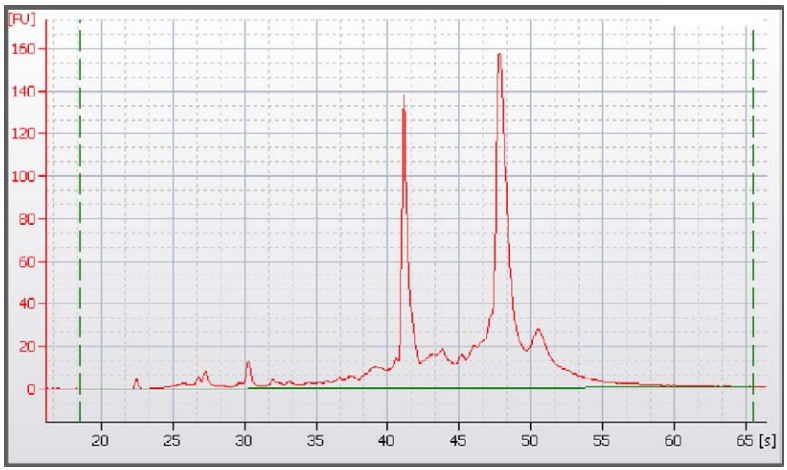
This protocol is optimized for 0.1–4 µg of total RNA. Lower amounts may result in inefficient ligation and low yield. The protocol has been tested using 0.1–10 µg of high-quality universal human reference total RNA as input. Use of RNA from other species, tissues, or qualities may require further optimization with regard to the initial input amount.

The protocol recommends diluting the in-line controls for tracking the steps involved in converting dsDNA into libraries. The dilution is optimized for 0.1–4 µg of high quality input RNA. When using less RNA or RNA with very low mRNA content, these controls may need further dilution. If no controls are added, use Resuspension Buffer in place of the controls in the protocol.

It is very important to use high-quality RNA as the starting material. 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 with an RNA Integrity Number (RIN) value greater than or equal to 8. RNA that has DNA contamination will result in an underestimation of the amount of RNA used. Illumina recommends including a DNase step with the RNA isolation method. However, contaminant DNA will be removed during mRNA purification.

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

Figure 6 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 that should be twice the intensity of the 18S rRNA band at 1.9 kb. Both kb determinations are relative to a RNA 6000 ladder. The mRNA will appear 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 0.1–4 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:

- For LT processing, step 11 of *Make RFP* on page 44.
- For HT processing, step 13 of *Make RFP* on page 80.

Positive Control

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

In-Line Control DNA

The End Repair Control, A-Tailing Control, and Ligation Control reagents contain DNA fragments used as controls for the enzymatic activities of the 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. Readout is determined by sequencing. If the sequence of an in-line control appears in the final sequencing data, it indicates that its corresponding step was successful. If it does not, or if it appears in substantially diminished numbers, it indicates the step failed. The controls are intended for troubleshooting and are useful for identifying the specific mode of failure, but are uninformative in cases where sequencing data is not generated from a library.

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

The control molecules work through the design of their ends (Table 3). Controls are added to the reactions just prior to their corresponding step in the protocol. Their end structures match those of a DNA molecule that has not gone through the step. If the step is successful, the control molecule will be modified to participate in downstream reactions of library generation and resulting in sequencing data. If the step fails, the control molecule will not go forward in the process and no sequencing data will be generated. Using 1 µg of starting material, the controls yield approximately 0.2% of clusters, although this can vary based on library yield.

Table 3 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 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 adapters to inserts	Ligation Control	Single-base 3' 'A' base overhang

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

The control reagents can be used for a variety of library insert sizes. Each is provided in ladders ranging from approximately 150–850 bp in 100 bp increments. Each control molecule has a unique DNA sequence, indicating both its function and size. The RTA software (version 1.9 and higher) recognizes these sequences and isolates the control sequences from the main body of sequencing reads and reports their counts per lane in the controls tab of the RTA status.html page.

Tracking Tools

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

- **Experienced User Cards** to guide you through the protocol, but with less detail than provided in this user guide.
- **Lab Tracking Forms** to record lab equipment start and stop times and record the barcode of each reagent and plate used in the protocol.
- **Sample Sheet Template** to record information about your samples for later use in data analysis.

NOTE
All of these documents can be downloaded via http://www.illumina.com/support/documentation.ilmn.

Lab Tracking Form

Create a copy of the Lab Tracking Form for each run. Use it to track information about your sample preparation such as operator information, start and stop times, reagent lot numbers, and barcodes. This form can be filled out and saved online or printed and filled in by hand.

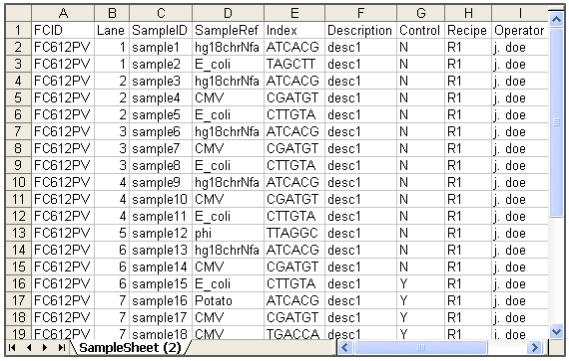
Sample Sheet

The sample sheet is a file that describes the samples in each lane, including the indexes used, and is required for demultiplexing following sequencing. For instructions on using the sample sheet to direct demultiplexing, see the analysis pipeline documentation.

The sample sheet is a comma-separated values (*.csv) file that contains the sample name and related information, as shown below. Create the sample sheet using Excel or another text editing tool that supports .csv files. Fill in your sample sheet according to the guidelines provided in this section.

Include lanes with multiplexed samples in the sample sheet, listing the information below. Lanes with a single sample can be left out of the sample sheet. These single sample lanes can then be aligned to a reference genome, as specified in the CASAVA config.template.txt file.

Figure 7 Example: Sample Sheet



	A	B	C	D	E	F	G	H	I
1	FCID	Lane	SampleID	SampleRef	Index	Description	Control	Recipe	Operator
2	FC612PV	1	sample1	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
3	FC612PV	1	sample2	E_coli	TAGCTT	desc1	N	R1	j. doe
4	FC612PV	2	sample3	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
5	FC612PV	2	sample4	CMV	CGATGT	desc1	N	R1	j. doe
6	FC612PV	2	sample5	E_coli	CTTGTA	desc1	N	R1	j. doe
7	FC612PV	3	sample6	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
8	FC612PV	3	sample7	CMV	CGATGT	desc1	N	R1	j. doe
9	FC612PV	3	sample8	E_coli	CTTGTA	desc1	N	R1	j. doe
10	FC612PV	4	sample9	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
11	FC612PV	4	sample10	CMV	CGATGT	desc1	N	R1	j. doe
12	FC612PV	4	sample11	E_coli	CTTGTA	desc1	N	R1	j. doe
13	FC612PV	5	sample12	phi	TTAGGC	desc1	N	R1	j. doe
14	FC612PV	6	sample13	hg18chrNfa	ATCACG	desc1	N	R1	j. doe
15	FC612PV	6	sample14	CMV	CGATGT	desc1	N	R1	j. doe
16	FC612PV	6	sample15	E_coli	CTTGTA	desc1	Y	R1	j. doe
17	FC612PV	7	sample16	Potato	ATCACG	desc1	Y	R1	j. doe
18	FC612PV	7	sample17	CMV	CGATGT	desc1	Y	R1	j. doe
19	FC612PV	7	sample18	CMV	TGACCA	desc1	Y	R1	j. doe

SampleSheet (2)

The sample sheet has the following fields:

Table 4 Sample Sheet Fields

Column Header	Description
FCID	The flow cell ID
Lane	A positive integer indicating the lane number (1–8)
Sample ID	The sample ID. This can be used to specify samples in the CASAVA config.template.txt file.
Sample Ref	The reference sequence for the sample. This can be used to specify a reference genome in the CASAVA config.template.txt.
Index	The index sequence
Description	The sample description
Control	Y indicates the lane is a control lane N indicates a sample
Recipe	The recipe used during sequencing
Operator	The name or ID of the operator

NOTE

To avoid misidentifying samples, ensure that the sample IDs entered in the sample sheet correctly correspond to the RNA samples used.

Adapter Index Sequences

The following table lists the TruSeq RNA Sample Prep v2 adapter index sequences.

NOTE

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

Table 5 TruSeq RNA Sample Prep v2 Adapter Index Sequences

Indexed Adapter	Sequence	Indexed Adapter	Sequence
AR001	ATCACG(A)	AR013	AGTCAA(C)
AR002	CGATGT(A)	AR014	AGTTCC(G)
AR003	TTAGGC(A)	AR015	ATGTCA(G)
AR004	TGACCA(A)	AR016	CCGTCC(C)
AR005	ACAGTG(A)	AR018	GTCCGC(A)
AR006	GCCAAT(A)	AR019	GTGAAA(C)
AR007	CAGATC(A)	AR020	GTGGCC(T)
AR008	ACTTGA(A)	AR021	GTTTCG(G)
AR009	GATCAG(A)	AR022	CGTACG(T)
AR010	TAGCTT(A)	AR023	GAGTGG(A)
AR011	GGCTAC(A)	AR025	ACTGAT(A)
AR012	CTTGTA(A)	AR027	ATTCCT(T)

Kit Contents

Check to ensure that you have all of the reagents identified in this section before proceeding. Each TruSeq RNA Sample Prep Kit v2 can be used to process up to 48 samples. Set A and B differ in the index pooling level.

- TruSeq RNA Sample Prep Kit v2 - Set A (48rxn), catalog # RS-122-2001
- TruSeq RNA Sample Prep Kit v2 - Set B (48rxn), catalog # RS-122-2002

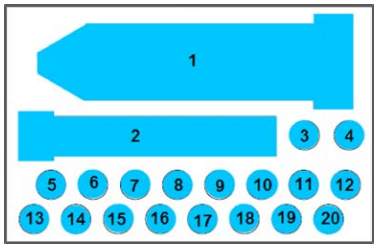
Kit Contents, Boxes A and B

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

Store at -15° to -25°C

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

Figure 8 TruSeq RNA Sample Prep Kit v2, Box A



1 Resuspension Buffer (RSB)

2 End Repair Mix (ERP)

3 A-Tailing Mix (ATL)

4 Ligation Mix (LIG)

5 End Repair Control (CTE)

6 A-Tailing Control (CTA)

7 Ligation Control (CTL)

8 Stop Ligation Buffer (STL)

9 RNA Adapter Index 2(AR002)

10 RNA Adapter Index 4 (AR004)

11 RNA Adapter Index 5 (AR005)

12 RNA Adapter Index 6 (AR006)

13 RNA Adapter Index 7 (AR007)

14 RNA Adapter Index 12 (AR012)

15 RNA Adapter Index 13 (AR013)

16 RNA Adapter Index 14 (AR014)

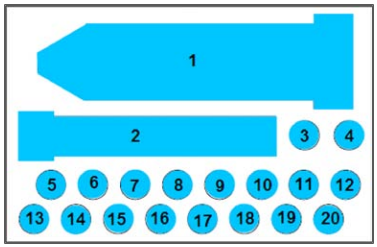
17 RNA Adapter Index 15 (AR015)

18 RNA Adapter Index 16 (AR016)

19 RNA Adapter Index 18 (AR018)

20 RNA Adapter Index 19 (AR019)

Figure 9 TruSeq RNA Sample Prep Kit v2, Box B



1 Resuspension Buffer (RSB)

2 End Repair Mix (ERP)

3 A-Tailing Mix (ATL)

4 Ligation Mix (LIG)

5 End Repair Control (CTE)

6 A-Tailing Control (CTA)

7 Ligation Control (CTL)

8 Stop Ligation Buffer (STL)

9 RNA Adapter Index 1 (AR001)

10 RNA Adapter Index 3 (AR003)

11 RNA Adapter Index 8 (AR008)

12 RNA Adapter Index 9 (AR009)

13 RNA Adapter Index 10 (AR010)

14 RNA Adapter Index 11 (AR011)

15 RNA Adapter Index 20 (AR020)

16 RNA Adapter Index 21 (AR021)

17 RNA Adapter Index 22 (AR022)

18 RNA Adapter Index 23 (AR023)

19 RNA Adapter Index 25 (AR025)

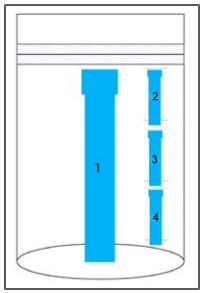
20 RNA Adapter Index 27 (AR027)

Kit Contents, Box 1

Store at 2° to 8°C

This box is shipped at room temperature on gel packs. As soon as you receive it, store the contents at 2° to 8°C.

Figure 10 TruSeq RNA Sample Prep Kit v2, Box 1



1 RNA Purification Beads (RPB)

2 CTE Dilution Tube, For Automated Use Only

3 CTA Dilution Tube, For Automated Use Only

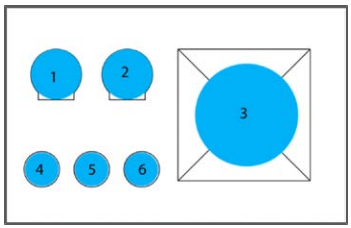
4 CTL Dilution Tube, For Automated Use Only

Kit Contents, Box 2

Store at -15° to -25°C

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

Figure 11 TruSeq RNA Sample Prep Kit v2 Box 2



1 Bead Binding Buffer (BBB)

2 Elution Buffer (ELB)

3 Bead Washing Buffer (BWB)

4 Elute, Prime, Fragment Mix (EPF)

5 First Strand Master Mix (FSM)

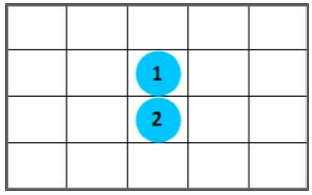
6 Second Strand Master Mix (SSM)

Kit Contents, PCR Prep Box

Store at -15° to -25°C

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

Figure 12 TruSeq RNA Sample Prep Kit v2, PCR Prep Box



1 PCR Master Mix (PMM)

2 PCR Primer Cocktail (PPC)

Consumables and Equipment

Check to ensure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation. These consumables and equipment are Illumina recommended for the TruSeq RNA Sample Preparation v2 protocols. The requirement of some supplies are dependent upon the protocol performed (LT or HT) and these items are specified in separate tables below.

Table 6 User-Supplied Consumables

Consumable	Supplier
1.5 ml RNase/DNase-free non-sticky tubes	Ambion, 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
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

Consumable	Supplier
MicroTube (6x16mm), AFA fiber with crimp-cap (Optional - for alternative fragmentation only)	Covaris, part # 520052
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma Aldrich, part # E7023
Microseal 'B' adhesive seals	BioRad, part # MSB-1001
MinElute Gel Extraction Kit (Optional - if starting with previously isolated mRNA)	QIAGEN, part# 28604
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
RNase/DNase-free 8-well PCR strip tubes and caps	General lab supplier
RNase/DNase zapper (to decontaminate surfaces)	General lab supplier
SuperScript II Reverse Transcriptase	Invitrogen, part # 18064-014
Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20	General lab supplier
Tween 20	Sigma, part # P7949
Ultra pure water	General lab supplier

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

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

Table 8 User-Supplied Consumables - Additional Items for HT Processing

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

Table 9 User-Supplied Equipment

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

Table 10 User-Supplied Equipment - Additional Items for HT Processing

Consumable	Supplier
High Speed Micro Plate Shaker	VWR, catalog # 13500-890 (110V/120V) VWR, catalog # 14216-214 (230V)
MIDI plate insert for heating system	Illumina, catalog # BD-60-601
Stroboscope	General lab supplier
Tru Temp Microheating System	Illumina, catalog # SC-60-503 (115V) Illumina, catalog # SC-60-504 (220V)

Chapter 3

Low-Throughput (LT) Protocol

Introduction

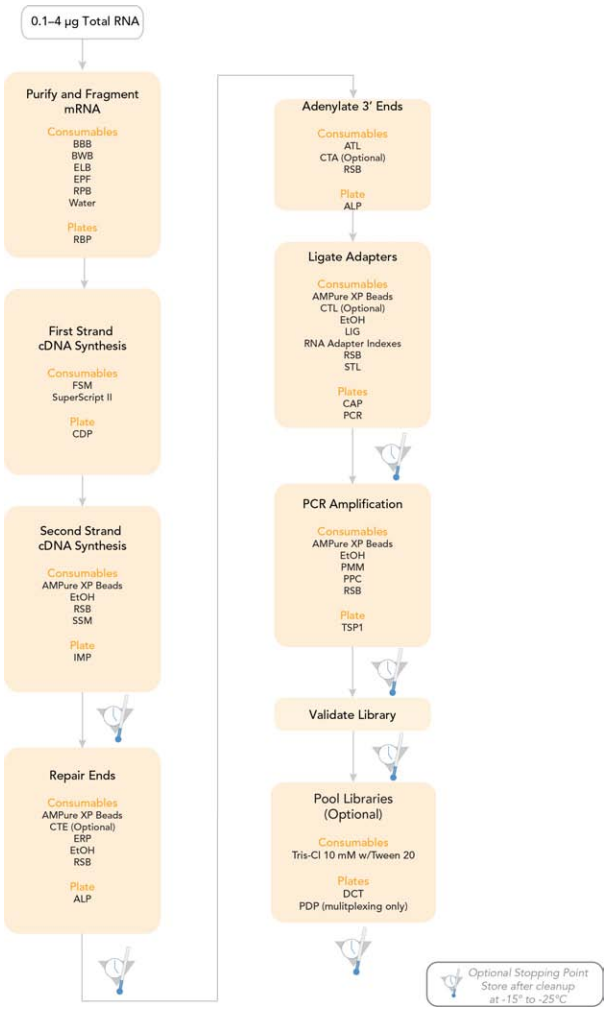
This chapter describes the TruSeq RNA Sample Preparation v2 low-throughput (LT) protocol. Illumina recommends this protocol for processing 48 or fewer samples. Follow the protocol in the order shown. For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample preparation.

When processing more than 48 samples, Illumina recommends following the protocol described in Chapter 4 High-Throughput (HT) Protocol.

Sample Prep Workflow

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

Figure 13 TruSeq RNA Sample Preparation v2 LT Workflow

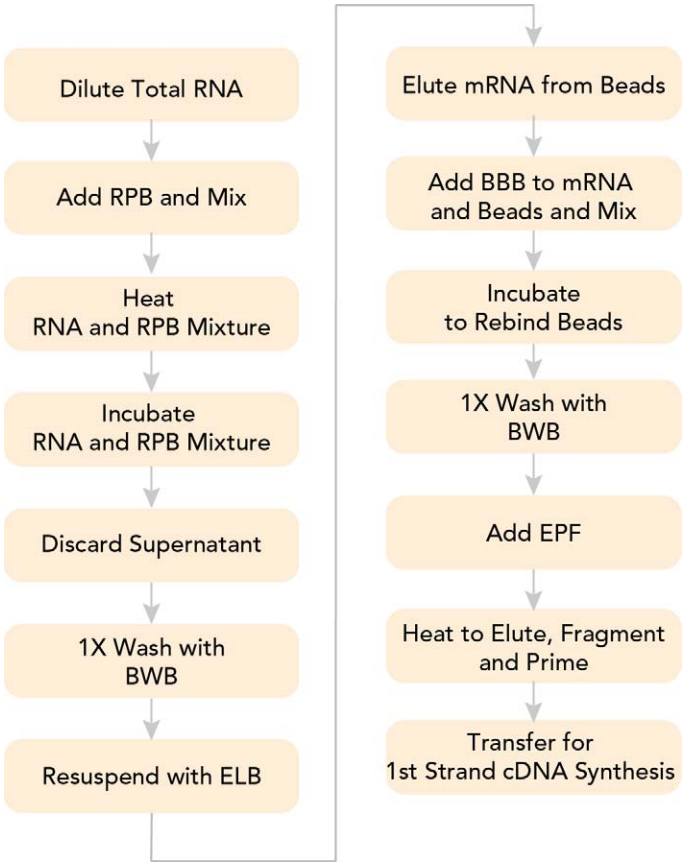


Purify and Fragment mRNA

This process purifies the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads using two rounds of purification. During the second elution of the poly-A RNA, the RNA is also fragmented and primed for cDNA synthesis.

Reference the following diagram while performing the purification procedures:

Figure 14 TruSeq RNA Sample Prep v2 Purification Workflow



It is important to follow this procedure exactly to ensure 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 0.1–4 µg of total RNA and use PCR plates with a magnetic plate stand for this process. Alternatively, you can start the protocol with 10–400 ng of previously isolated mRNA. If you do so, first perform the following procedure:

1. Ethanol precipitate the mRNA or concentrate with a Qiagen MinElute column to ≤ 5 µl with water.
2. Resuspend the pellet in 18 µl Elute, Prime, Fragment Mix or add 13 µl Elute, Prime, Fragment Mix to 5 µl mRNA in water.
3. Heat the mRNA to fragment at *Incubate RFP* on page 45 in this process.

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

Illumina-Supplied Consumables

- Bead Binding Buffer (BBB) (1 tube per 48 reactions)
- Bead Washing Buffer (BWB) (1 tube per 48 reactions)
- Elution Buffer (ELB) (1 tube per 48 reactions)
- Elute, Prime, Fragment Mix (EPF) (1 tube per 48 reactions)
- RNA Purification Beads (RPB) (1 tube per 48 reactions)
- RBP (RNA Bead Plate) barcode label

User-Supplied Consumables

- 96-well 0.3 ml PCR plate
- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)
- Ultra Pure Water

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - Bead Binding Buffer
  - Bead Washing Buffer
  - Elution Buffer
  - Elute, Prime, Fragment Mix
- Remove the RNA Purification Beads tube from storage and let stand to bring to room temperature.
- Pre-heat the thermal cycler lid to 100°C.
- Pre-program the thermal cycler with the following programs:
  - 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**
- Set the centrifuge to 15° to 25°C, if refrigerated.
- Apply a RBP barcode label to a new 96-well 0.3 ml PCR plate.

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

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 thawed RNA Purification Beads tube vigorously to completely resuspend the oligo-dT beads.
3. Add 50 µl of RNA Purification Beads to each well of the RBP to bind the poly-A RNA to the oligo dT magnetic 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. Pre-heat the thermal cycler lid to 100°C.

2 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 poly-A RNA to the beads.

3 Remove the RBP plate from the thermal cycler when it reaches 4°C.

4 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 Place the RBP plate on the magnetic stand at room temperature for 5 minutes to separate the poly-A RNA bound beads from the solution.

2 Remove the adhesive seal from the RBP plate.

3 Remove and discard all of the supernatant from each well of the RBP plate. Take care not to disturb the beads. Change the tip after each sample.

4 Remove the RBP plate from the magnetic stand.

5 Wash the beads by adding 200 µl of 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. Change the tip after each sample.

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

7 Briefly centrifuge the thawed Elution Buffer to 600 xg for 5 seconds.

8 Remove and discard all of the supernatant from each well of the RBP plate. Take care not to disturb the beads. Change the tip after each sample.The supernatant contains the majority of the ribosomal and other non-messenger RNA.

9 Remove the RBP plate from the magnetic stand.

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

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

12 Store the Elution Buffer tube at 4°C.

Incubate 2 RBP

1. Pre-heat the thermal cycler lid to 100°C.
2. 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. This releases both the mRNA and any contaminant rRNA that has bound the beads non-specifically.
3. Remove the RBP plate from the thermal cycler when it reaches 25°C.
4. Place the RBP plate on the bench at room temperature and remove the adhesive seal from the plate.

Make RFP

1. Add 50 µl of 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. Change the tip after each sample.
2. Incubate the RBP plate at room temperature for 5 minutes and store the Bead Binding Buffer tube at 2° to 8°C.
3. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
4. Remove and discard all of the supernatant from each well of the RBP plate. Take care not to disturb the beads. Change the tip after each sample.
5. Remove the RBP plate from the magnetic stand.
6. Wash the beads by adding 200 µl of Bead Washing Buffer in each well of the RBP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly. Change the tip after each sample.
7. Store the Bead Washing Buffer tube at 2° to 8°C.
8. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
9. Remove and discard all of the supernatant from each well of the RBP plate. Take care not to disturb the beads. Change the tip after each sample.The supernatant

contains residual rRNA and other contaminants that were released in the first elution and did not rebind the beads.

10 Remove the RBP plate from the magnetic stand.

11 Add 19.5 µl of Elute, Prime, Fragment Mix to each well of the RBP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly. Change the tip after each sample. The Elute, Prime, Fragment Mix contains random hexamers for RT priming and serves as the 1st strand cDNA synthesis reaction buffer.

12 Seal the RBP plate with a Microseal 'B' Adhesive seal.

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

Incubate RFP

1 Pre-heat the thermal cycler lid to 100°C.

2 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.

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

4 Proceed immediately to *Synthesize First Strand cDNA* on page 46.

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.

Illumina-Supplied Consumables

- CDP (cDNA Plate) barcode label
- First Strand Master Mix (FSM) (1 tube)

User-Supplied Consumables

- 96-well 0.3 ml PCR plate
- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)
- SuperScript II Reverse Transcriptase

Preparation

- Remove one tube of First Strand Master Mix from -15° to -25°C storage and thaw it at room temperature.
- Pre-program the thermal cycler with the following program and save as **1st Strand**:
  - 25°C for 10 minutes
  - 42°C for 50 minutes
  - 70°C for 15 minutes
  - Hold at 4°C
- Pre-heat the thermal cycler lid to 100°C.
- Apply a CDP barcode label to a new 96-well 0.3 ml PCR plate.

NOTE
This process has been designed to generate a 1st strand master mix containing the SuperScript II reverse transcriptase that is stable to additional freeze thaw cycles and can be used for subsequent experiments.

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. Briefly centrifuge the thawed First Strand Master Mix tube to 600 xg for 5 seconds.
5. Add 50 µl SuperScript II to the First Strand Master Mix tube (ratio: 1 µl SuperScript II for each 79.6 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 of 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. Change the tip after each sample.
7. Seal the CDP plate with a Microseal 'B' Adhesive seal and centrifuge briefly.
8. Return the First Strand Master Mix tube back to -15° to -25°C storage immediately after use.

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 and thaw cycles are anticipated, divide the First Strand Master Mix into smaller aliquots and store at -15° to -25°C.

Incubate 1 CDP

1. Pre-heat the thermal cycler lid to 100°C.
2. Incubate the CDP plate on the thermal cycler, with the lid closed, using the **1st Strand** program:
   a. 25°C for 10 minutes

b 42°C for 50 minutes
c 70°C for 15 minutes
d Hold at 4°C

3 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 49.

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 2nd strand reaction mix.

Illumina-Supplied Consumables

- Resuspension Buffer (RSB) (1 tube)
- Second Strand Master Mix (SSM) (1 tube)
- IMP (Insert Modification Plate) barcode label

User-Supplied Consumables

- 96-well 0.3 ml PCR plate
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)

Preparation

- Remove one tube each of Second Strand Master Mix and Resuspension Buffer from -15° to -25°C storage and thaw it at room temperature.
- Review *AMPure XP Handling* on page 10.
- 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 IMP barcode label to a new 96-well 0.3 ml PCR plate.

Add SSM

1. Briefly centrifuge the thawed Second Strand Master Mix to 600 xg for 5 seconds.
2. Remove the adhesive seal from the CDP plate.

3 Add 25 µl of 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. Change the tip after each sample.

4 Seal the CDP plate with a Microseal 'B' Adhesive seal.

Incubate 2 CDP

1 Incubate the CDP plate on the pre-heated thermal cycler, with the lid closed, at 16°C for 1 hour.

2 Remove the CDP plate from the thermal cycler, remove the adhesive seal, and let stand to bring the plate to room temperature.

Clean Up CDP

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 when working with AMPure XP Beads.

1 Vortex the AMPure XP beads until they are well dispersed, then add 90 µl of well-mixed AMPure XP beads to each well of the CDP plate containing 50 µl of ds cDNA. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

3 Place the CDP plate on the magnetic stand at room temperature, for 5 minutes to ensure that all of the beads are bound to the side of the wells.

4 Remove and discard 135 µl of the supernatant from each well of the CDP plate. Take care not to disturb the beads. Change the tip after each sample.

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

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

6 Incubate the CDP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tip after each sample.

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

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

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

10 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.

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

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

13 Transfer 50 µl of the supernatant (ds cDNA) from the CDP plate to the new 0.3 ml PCR plate labeled with the IMP barcode.

SAFE STOPPING POINT

If you do not plan to proceed to *Perform End Repair* on page 52 immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

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.

Illumina-Supplied Consumables

- (Optional) End Repair Control (CTE) (1 tube per 48 reactions)
- End Repair Mix (ERP) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)
- ALP (Adapter Ligation Plate) barcode label

User-Supplied Consumables

- 96-well 0.3 ml PCR plate
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)

Preparation

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

  NOTE
  The use of the End Repair Control is optional and it can be replaced with the same volume of Resuspension Buffer.
- Review *AMPure XP Handling* on page 10.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the thermal cycler to 30°C.
- Apply a 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:
     - Briefly centrifuge the thawed End Repair Control tube to 600 xg 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 of diluted End Repair Control to each well of the IMP plate that contains 50 µl of ds cDNA. Change the tip after each sample.
   - If not using the in-line control reagent, add 10 µl of Resuspension Buffer to each well of the IMP plate that contains 50 µl of ds cDNA. Change the tip after each sample.
2. Add 40 µl of End Repair Mix to each well of the IMP plate containing the ds cDNA. Change the tip after each sample.
3. Adjust the pipette to 100 µl, then gently pipette the entire volume of each pooled library up and down 10 times to mix thoroughly. Change the tip after each sample.
4. Seal the IMP plate with a Microseal 'B' adhesive seal.

Incubate 1 IMP

1. Pre-heat the thermal cycler lid to 100°C.
2. Incubate the IMP plate on the pre-heated thermal cycler, with the lid closed, for 30 minutes at 30°C.
3. Remove the IMP plate from the thermal cycler.

Clean Up IMP

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 when working with AMPure XP Beads.

1. Remove the adhesive seal from the IMP plate.

2 Vortex the AMPure XP Beads until they are well dispersed, then add 160 µl well-mixed AMPure XP Beads to each well of the IMP plate containing 100 µl of End Repair Mix.

3 Adjust the pipette to 200 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

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 appears clear.

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

7 Repeat step 6 once.

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 of freshly prepared 80% EtOH to each well with a sample without disturbing the beads.

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

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

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

12 Resuspend the dried pellet in each well with 17.5 µl Resuspension Buffer. 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 appears clear.

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

SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3′ Ends* on page 56 immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Adenylate 3' Ends

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

Illumina-Supplied Consumables

- (Optional) A-Tailing Control (CTA) (1 tube per 48 reactions)
- A-Tailing Mix (ATL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB)

User-Supplied Consumables

- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - A-Tailing Control or Resuspension Buffer
  - A-Tailing Mix

  NOTE
  The use of the A-Tailing Control is optional and it can be replaced with the same volume of Resuspension Buffer.
- Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up IMP* on page 53 and let stand to thaw at room temperature.
  - Briefly centrifuge the thawed ALP plate to 280 xg for 1 minute
  - Remove the adhesive seal from the ALP plate.
- Pre-heat the thermal cycler to 37°C.

Add ATL

1. Do one of the following:
   - If using the in-line control reagent:
     - Briefly centrifuge the thawed A-Tailing Control tube to 600 xg for 5 seconds.
     - Dilute the A-Tailing Control to 1/100 in Resuspension Buffer (1 µl A-Tailing Control + 99 µl Resuspension Buffer) before use. Discard the diluted A-Tailing Control after use.
     - Add add 2.5 µl of diluted A-Tailing Control to each well of the ALP plate.
   - If not using the in-line control reagent, add 2.5 µl of Resuspension Buffer to each well of the ALP plate.
2. Add 12.5 µl of thawed A-Tailing Mix to each well of the ALP plate.
3. Adjust the pipette to 30 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.
4. Seal the ALP plate with a Microseal 'B' adhesive seal.

Incubate 1 ALP

1. Pre-heat the thermal cycler lid to 100°C.
2. Incubate the ALP plate on the pre-heated thermal cycler, with the lid closed, for 30 minutes at 37°C.
3. Immediately remove the ALP plate from the thermal cycler, then proceed immediately to *Ligate Adapters* on page 58.

Ligate Adapters

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

Illumina-Supplied Consumables

- Ligation Mix (LIG) (1 tube per 48 reactions)
- (Optional) Ligation Control (CTL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB)
- RNA Adapter Indexes (AR001–AR016, AR018–AR023, AR025, AR027) (1 tube per column of 8 reactions, depending on the RNA Adapter Indexes being used)
- Stop Ligation Buffer (STL) (1 tube per 48 reactions)
- CAP (Clean Up ALP Plate) barcode label
- PCR (Polymerase Chain Reaction) barcode label

User-Supplied Consumables

- 96-well 0.3 ml PCR plates (2)
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seals
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - Appropriate RNA Adapter Index tubes, depending on the RNA Adapter Indexes being used
  - Stop Ligation Buffer
  - Ligation Control or Resuspension Buffer

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

- Review *AMPure XP Handling* on page 10.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the thermal cycler to 30°C.
- 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. Each column should contain a common index. This will facilitate pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.

Add LIG

1. Briefly centrifuge the appropriate/desired thawed RNA Adapter Index tubes, Ligation Control (if using Ligation Control), and Stop Ligation Buffer tubes to 600 xg for 5 seconds.
2. Immediately before use, remove the Ligation Mix tube from -15° to -25°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 add 2.5 µl of thawed Ligation Control to each well of the ALP plate.
   - If not using the in-line control reagent, add 2.5 µl of Resuspension Buffer to each well of the ALP plate.
5. Add 2.5 µl of Ligation Mix to each well of the ALP plate.
6. Return the Ligation Mix tube back to -15° to -25°C storage immediately after use.
7. Add 2.5 µl of the appropriate/desired thawed RNA Adapter Index to each well of the ALP plate.

8 Adjust the pipette to 37.5 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

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

Incubate 2 ALP

1 Pre-heat the thermal cycler lid to 100°C.

2 Incubate the ALP plate on the pre-heated thermal cycler, with the lid closed, at 30°C for 10 minutes.

3 Remove the ALP plate from the thermal cycler.

Add STL

1 Remove the adhesive seal from the ALP plate.

2 Add 5 µl of Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation.

3 Adjust the pipette to 42.5 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

Clean Up ALP

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 when working with AMPure XP Beads.

1 Vortex the AMPure XP Beads until they are well dispersed, then add 42 µl of mixed AMPure XP Beads to each well of the ALP plate.

2 Adjust the pipette to 85 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

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

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

5 Remove and discard 79.5 µl of the supernatant from each well of the ALP plate. Change the tip after each sample.

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

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

7 Incubate the ALP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well. Change the tip after each sample.

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

9 While keeping the ALP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes and then remove the plate from the magnetic stand.

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

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

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

13 Transfer 50 µl of the clear supernatant from each well of the ALP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CAP barcode. Change the tip after each sample.

14 Vortex the AMPure XP Beads until they are well dispersed, then add 50 µl of mixed AMPure XP Beads to each well of the CAP plate for a second clean up.

15 Adjust the pipette to 100 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly. Change the tip after each sample.

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

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

18 Remove and discard 95 µl of the supernatant from each well of the CAP plate. Change the tip after each sample.

NOTE

Leave the CAP plate on the magnetic stand while performing the following 80% EtOH wash steps (19–21)

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

20 Incubate the CAP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well. Change the tip after each sample.

21 Repeat steps 19 and 20 once for a total of two 80% EtOH washes.

22 While keeping the CAP plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes and then remove the plate from the magnetic stand.

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

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

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

SAFE STOPPING POINT

If you do not plan to proceed to *Enrich DNA Fragments* on page 63 immediately, the protocol can be safely stopped here. If you are stopping, seal the CAP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Enrich DNA Fragments

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

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

Illumina-Supplied Consumables

- PCR Master Mix (PMM) (1 tube per 48 reactions)
- PCR Primer Cocktail (PPC) (1 tube per 48 reactions)
- Resuspension Buffer (RSB)
- TSP1 (Target Sample Plate) barcode label

User-Supplied Consumables

- 96-well 0.3 ml PCR plate
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seals
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)

Preparation

- Remove one tube each of PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature. Once thawed, keep the tubes on ice.
- Briefly centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.

- Review *AMPure XP Handling* on page 10.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the PCR plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 60 and let stand to thaw at room temperature.
  - Briefly centrifuge the thawed PCR plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed PCR plate.
- Pre-program the thermal cycler with the following program and save as **PCR**:
  - 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
- Pre-heat the thermal cycler lid to 100°C.
- Apply a TSP1 barcode label to a new 96-well 0.3 ml PCR plate.

Make PCR

1. Add 5 µl of thawed PCR Primer Cocktail to each well of the PCR plate. Change the tip after each sample.
2. Add 25 µl of thawed PCR Master Mix to each well of the PCR plate. Change the tip after each sample.
3. Adjust the pipette to 40 µl, then gently pipette the entire volume up and down 10 times to mix thoroughly.
4. Seal the PCR plate with a Microseal 'B' adhesive seal.

Amp PCR

1. Pre-heat the thermal cycler lid to 100°C.
2. Amplify the PCR plate in the pre-programmed thermal cycler, with the lid closed, as follows:
   a. 98°C for 30 seconds

b 15 cycles of:
   98°C for 10 seconds
   60°C for 30 seconds
   72°C for 30 seconds
c 72°C for 5 minutes
d Hold at 10°C

Clean Up PCR

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 when working with AMPure XP Beads.

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

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

6. With the PCR plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.
7. Incubate the PCR plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well. Change the tip after each sample.
8. Repeat steps 6 and 7 once for a total of two 80% EtOH washes.
9. While keeping the PCR plate on the magnetic stand, let the samples air dry at room temperature for 15 minutes and then remove the plate from the magnetic stand.

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

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

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

13 Transfer 30 µl of the clear supernatant from each well of the PCR plate to the corresponding well of the new 0.3 ml PCR plate labeled with the TSP1 barcode. Change the tip after each sample.

SAFE STOPPING POINT

If you do not plan to proceed to *Validate Library* on page 67 immediately, the protocol can be safely stopped here.If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Validate Library

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

Quantify Libraries

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

Quality Control

1 Load 1 µl of the resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA specific chip such the Agilent DNA-1000.

2 Check the size and purity of the sample. The final product should be a band at approximately 260 bp (for single-read libraries).

Figure 15 Example of TruSeq RNA Sample Prep v2 Library Size Distribution

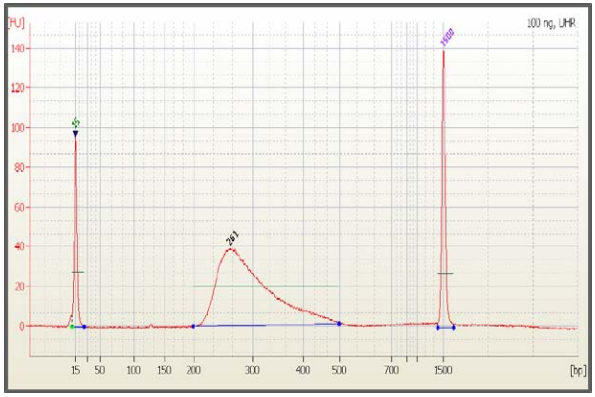
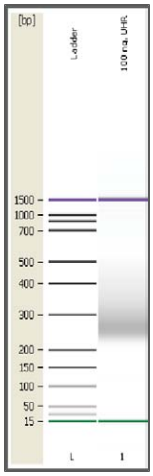


Figure 16 TruSeq RNA Sample Prep v2 260 bp PCR Product



Normalize and Pool Libraries (Optional)

This process describes how to prepare DNA templates that will be applied to cluster generation. Multiplexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. Non-multiplexed DNA libraries are normalized to 10 nM in the DCT plate without pooling.

Illumina-Supplied Consumables

- DCT (Diluted Cluster Template) barcode label
- PDP (Pooled DCT Plate) barcode label (for multiplexing only)

User-Supplied Consumables

- 96-well 0.3 ml PCR plate (for multiplexing only)
- 96-well MIDI plate
- Microseal 'B' Adhesive seals
- Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20

Preparation

- Apply a DCT barcode label to a new 96-well MIDI plate.
- Apply a PDP barcode label to a new 96-well 0.3 ml PCR plate (for multiplexing only).
- Remove the TSP1 plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 65, and let stand to thaw at room temperature.
  - Briefly centrifuge the thawed TSP1 plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.

Make DCT

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

NOTE
Depending on the yield quantification data of each sample library, the final volume in the DCT plate may 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-multiplexed paired-end libraries, the protocol stops here. Do one of the following:
  - Proceed to cluster generation. See the *Illumina Cluster Generation User Guide*.
  - Seal the DCT plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.
- For multiplexed paired-end libraries, proceed to Make PDP.

Make PDP (for multiplexing 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 should be 10X the number of combined sample libraries and will be 10–120 µl (1–12 libraries).

Table 11 Pooled Sample Volumes

Number of pooled samples	Volume (µl)
1	10
2	20
3	30
4	40

Number of pooled samples	Volume (µl)
5	50
6	60
7	70
8	80
9	90
10	100
11	110
12	120

NOTE
Keep track of which sample goes into which well, to 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. See the *Illumina Cluster Generation User Guide*.
- Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.

Chapter 4

High-Throughput (HT) Protocol

Introduction

This chapter describes the TruSeq RNA Sample Preparation v2 high-throughput (HT) protocol. Illumina recommends this protocol when processing more than 48 samples. Follow the protocols in the order shown. For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample preparation.

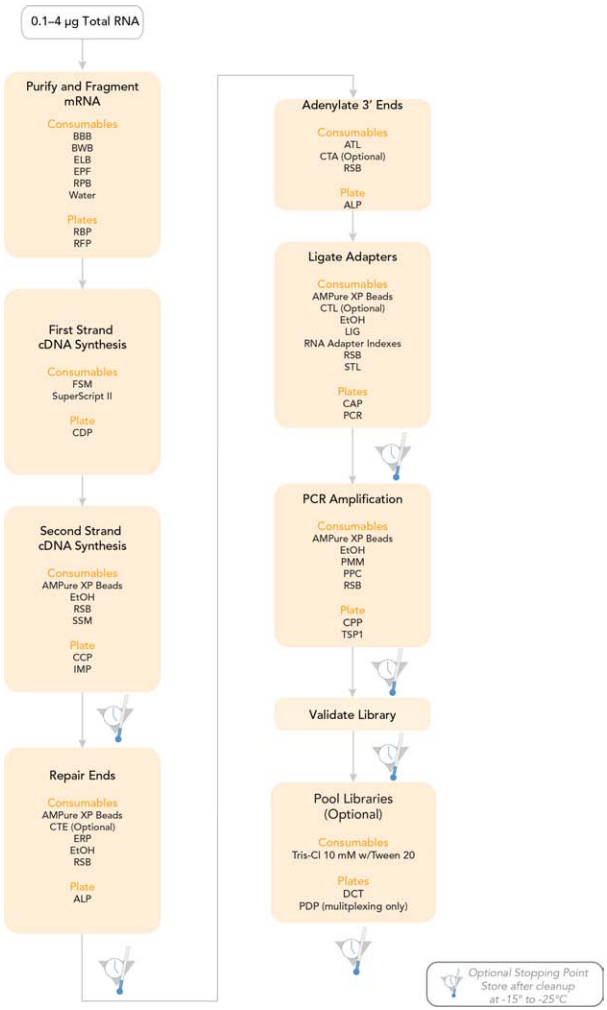
When processing 48 or fewer samples, Illumina recommends following the Chapter 3 Low-Throughput (LT) Protocol.

The HT protocol requires shaking and heating equipment to mix reagents and for incubation (see *Consumables and Equipment* on page 33).

Sample Prep Workflow

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

Figure 17 TruSeq RNA Sample Preparation v2 HT Workflow

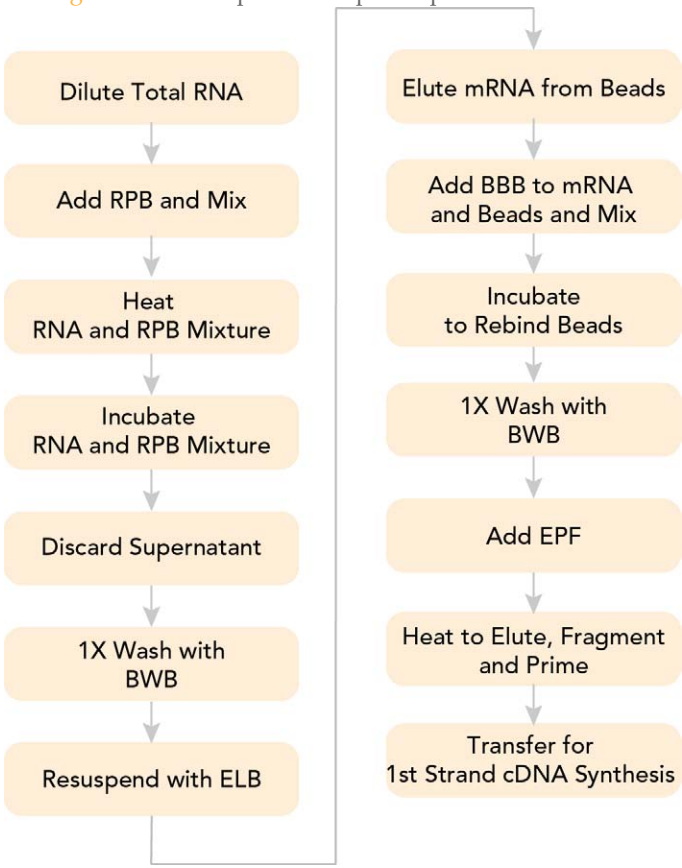


Purify and Fragment mRNA

This process purifies the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads using two rounds of purification. During the second elution of the poly-A RNA, the RNA is also fragmented and primed for cDNA synthesis.

Reference the following diagram while performing the purification procedures:

Figure 18 TruSeq RNA Sample Prep v2 Purification Workflow



It is important to follow this procedure exactly to ensure 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 0.1–4 µg of total RNA and use PCR plates with a magnetic plate stand for this process. Alternatively, you can start the protocol with 10–400 ng of previously isolated mRNA. If you do so, first perform the following procedure:

1. Ethanol precipitate the mRNA or concentrate with a Qiagen MinElute column to ≤ 5 µl with water.
2. Resuspend the pellet in 18 µl Elute, Prime, Fragment Mix or add 13 µl Elute, Prime, Fragment Mix to 5 µl mRNA in water.
3. Heat the mRNA to fragment at *Incubate RFP* on page 81 in this process.

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

Illumina-Supplied Consumables

- Bead Binding Buffer (BBB) (1 tube per 48 reactions)
- Bead Washing Buffer (BWB) (1 tube per 48 reactions)
- Elution Buffer (ELB) (1 tube per 48 reactions)
- Elute, Prime, Fragment Mix (EPF) (1 tube per 48 reactions)
- RNA Purification Beads (RPB) (1 tube per 48 reactions)
- RBP (RNA Bead Plate) barcode label
- RFP (RNA Fragmentation Plate) barcode label

User-Supplied Consumables

- 96-well HSP plate
- 96-well MIDI plate
- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)
- RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)
- Ultra Pure Water

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - Bead Binding Buffer
  - Bead Washing Buffer
  - Elution Buffer
  - Elute, Prime, Fragment Mix
- Remove the RNA Purification Beads tube from storage and let stand to bring to room temperature.
- Pre-heat the microheating system to 65°C.
- Pre-program the thermal cycler to 94°C for 8 minutes, 4°C hold and save as **Elution 2 - Frag - Prime**
- Pre-heat the thermal cycler lid to 100°C.
- Ensure that the microplate shaker is properly calibrated to 1,000 rpm using a stroboscope.
- Set the centrifuge to 15° to 25°C, if refrigerated.
- Apply a RBP barcode label to a new 96-well MIDI plate.
- Apply a RFP barcode label to a new 96-well HSP plate.

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

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 thawed RNA Purification Beads tube vigorously to completely resuspend the oligo-dT beads.
3. Add 50 µl of RNA Purification Beads to each well of the RBP plate to bind the poly-A RNA to the oligo dT magnetic 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 1,000 rpm for 1 minute.

Incubate 1 RBP

1. Place the sealed RBP plate on the pre-heated microheating system at 65°C for 5 minutes, with the lid closed, to denature the RNA and facilitate binding of the poly-A 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. Place the RBP plate on the magnetic stand at room temperature for 5 minutes to separate the poly-A RNA bound beads from the solution.
2. Remove the adhesive seal from the RBP plate.
3. Remove and discard all of the supernatant from each well of the RBP plate. Take care not to disturb the beads. Change the tip after each sample.
4. Remove the RBP plate from the magnetic stand.
5. Wash the beads by adding 200 µl of Bead Washing Buffer in each well of the RBP plate to remove unbound RNA. Change the tip after each sample. 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 1,000 rpm for 1 minute.
6. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
7. Remove the adhesive seal from the RBP plate.
8. Briefly centrifuge the thawed Elution Buffer to 600 xg for 5 seconds.
9. Remove and discard all of the supernatant from each well of the RBP plate. Take care not to disturb the beads. Change the tip after each sample.The supernatant contains the majority of the ribosomal and other non-messenger RNA.
10. Remove the RBP plate from the magnetic stand.

11 Add 50 µl of Elution Buffer in each well of the RBP plate. Change the tip after each sample. 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 1,000 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 at 80°C for 2 minutes, with the lid closed, to elute the mRNA from the beads.This releases both the mRNA and any contaminant rRNA that has bound the beads non-specifically.
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 and remove the adhesive seal from the plate.

Make RFP

1 Add 50 µl of 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. Change the tip after each sample. 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 1,000 rpm for 1 minute.
2 Incubate the RBP plate at room temperature for 5 minutes and store the Bead Binding Buffer tube at 2° to 8°C.
3 Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
4 Remove the adhesive seal from the RBP plate.
5 Remove and discard all of the supernatant from each well of the RBP plate. Take care not to disturb the beads. Change the tip after each sample.
6 Remove the RBP plate from the magnetic stand.

7 Wash the beads by adding 200 µl of Bead Washing Buffer in each well of the RBP plate. Change the tip after each sample. 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 1,000 rpm for 1 minute.

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

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

10 Remove the adhesive seal from the RBP plate.

11 Remove and discard all of the supernatant from each well of the RBP plate. Take care not to disturb the beads. Change the tip after each sample.The supernatant contains residual rRNA and other contaminants that were released in the first elution and did not rebind the beads.

12 Remove the RBP plate from the magnetic stand.

13 Add 19.5 µl of Elute, Prime, Fragment Mix to each well of the RBP plate. Change the tip after each sample. The Elute, Prime, Fragment Mix contains random hexamers for RT priming and serves as the 1st 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 1,000 rpm for 1 minute.

14 Remove the adhesive seal from the RBP plate.

15 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. Change the tip after each sample.

16 Seal the RFP plate with a Microseal 'B' Adhesive seal.

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

Incubate RFP

1 Pre-heat the thermal cycler lid to 100°C.

2 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.

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

4 Proceed immediately to *Synthesize First Strand cDNA* on page 83.

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.

Illumina-Supplied Consumables

- CDP (cDNA Plate) barcode label
- First Strand Master Mix (FSM) (1 tube)

User-Supplied Consumables

- 96-well HSP plate
- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs
- RNase/DNase-free Strip Tubes and Caps
- SuperScript II Reverse Transcriptase

Preparation

- Remove one tube of First Strand Master Mix from -15° to -25°C storage and thaw it at room temperature.
- Pre-program the thermal cycler with the following program and save as **1st Strand**:
  - 25°C for 10 minutes
  - 42°C for 50 minutes
  - 70°C for 15 minutes
  - Hold at 4°C
- Pre-heat the thermal cycler lid to 100°C.
- Ensure that the microplate shaker is properly calibrated to 1,000 rpm using a stroboscope.
- Apply a CDP barcode label to a new 96-well HSP plate.

NOTE
This process has been designed to generate a 1st strand master mix containing the SuperScript II reverse transcriptase that is stable to additional freeze thaw cycles and can be used for subsequent experiments

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. Briefly centrifuge the thawed First Strand Master Mix tube to 600 xg for 5 seconds.
5. Add 50 µl SuperScript II to the First Strand Master Mix tube (ratio: 1 µl SuperScript II for each 79.6 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 of First Strand Master Mix and SuperScript II mix to each well of the CDP plate. Change the tip after each sample. 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 1,600 rpm for 20 seconds.
7. Return the First Strand Master Mix tube back to -15° to -25°C storage immediately after use.

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 and thaw cycles are anticipated, divide the First Strand Master Mix into smaller aliquots and store at -15° to -25°C.

Incubate 1 CDP

1. Pre-heat the thermal cycler lid to 100°C.
2. Incubate the CDP plate on the thermal cycler, with the lid closed, using the **1st Strand** program:
   a. 25°C for 10 minutes

b 42°C for 50 minutes
c 70°C for 15 minutes
d Hold at 4°C

3 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 86.

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 2nd strand reaction mix.

Illumina-Supplied Consumables

- Resuspension Buffer (RSB) (1 tube)
- Second Strand Master Mix (SSM) (1 tube)
- CCP (cDNA Clean Up Plate) barcode label
- IMP (Insert Modification Plate) barcode label

User-Supplied Consumables

- 96-well MIDI plates (2)
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs
- RNase/DNase-free Strip Tubes and Caps

Preparation

- Remove one tube each of Second Strand Master Mix and Resuspension Buffer from -15° to -25°C storage and thaw it at room temperature.
- Review *AMPure XP Handling* on page 10.
- 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 a IMP barcode label to a new 96-well MIDI plate.

Add SSM

1. Briefly centrifuge the thawed Second Strand Master Mix to 600 xg for 5 seconds.
2. Remove the adhesive seal from the CDP plate.

3 Add 25 µl of thawed Second Strand Master Mix to each well of the CDP plate. Change the tip after each sample. 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 1,600 rpm for 20 seconds.

Incubate 2 CDP

1 Incubate the CDP plate on the pre-heated thermal cycler, with the lid closed, at 16°C for 1 hour.

2 Remove the CDP plate from the thermal cycler, remove the adhesive seal, and let stand to bring the plate to room temperature.

Clean Up CDP

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 when working with AMPure XP Beads.

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

2 Transfer the entire contents from each well of the CDP plate to the corresponding well of the CCP plate containing AMPure XP beads. Change the tip after each sample. Mix thoroughly as follows:
   a Seal the CCP plate with a Microseal 'B' Adhesive seal.
   b Shake the CCP plate on a microplate shaker at 1,800 rpm for 2 minutes.

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

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

5 Remove the adhesive seal from the CCP plate.

6 Remove and discard 135 µl of the supernatant from each well of the CCP plate. Take care not to disturb the beads. Change the tip after each sample.

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

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

8 Incubate the CCP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tip after each sample.

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

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

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

12 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 1,800 rpm for 2 minutes.
   c Centrifuge the CCP plate to 280 xg for 1 minute.

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

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

15 Remove the adhesive seal from the CCP plate.

16 Transfer 50 µl of the 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 to *Perform End Repair* on page 89 immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

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.

Illumina-Supplied Consumables

- (Optional) End Repair Control (CTE) (1 tube per 48 reactions)
- End Repair Mix (ERP) (1 tube per 48 reactions)
- Resuspension Buffer (RSB) (1 tube)
- ALP (Adapter Ligation Plate) barcode label

User-Supplied Consumables

- 96-well MIDI plate
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs
- RNase/DNase-free Strip Tubes and Caps

Preparation

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

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

- Review *AMPure XP Handling* on page 10.
- 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.
- Calibrate the microplate shaker with a stroboscope and set it to 1,800 rpm.
- Apply a 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:
  - Briefly centrifuge the thawed End Repair Control tube to 600 xg 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 of diluted End Repair Control to each well of the IMP plate that contains 50 µl of ds cDNA. Change the tip after each sample.
- If not using the in-line control reagent, add 10 µl of Resuspension Buffer to each well of the IMP plate that contains 50 µl of ds cDNA. Change the tip after each sample.

2 Add 40 µl of 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 1,800 rpm for 2 minutes.
   c Centrifuge the IMP plate to 280 xg for 1 minute.

Incubate 1 IMP

1 Incubate the IMP plate on the pre-heated microheating system, with the lid closed, for 30 minutes at 30°C.

2 Remove the IMP plate from the microheating system.

Clean Up IMP

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 when working with AMPure XP Beads.

1 Remove the adhesive seal from the IMP plate.

2 Vortex the AMPure XP Beads until they are well dispersed, then add 160 µl well-mixed AMPure XP Beads to each well of the IMP plate containing 100 µl of 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 1,800 rpm for 2 minutes.

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

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

5 Remove the adhesive seal from the IMP plate.

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

7 Repeat step 6 once.

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 of freshly prepared 80% EtOH to each well with a sample without disturbing the beads.

9 Incubate the IMP plate at room temperature for 30 seconds, then remove and discard all of the supernatant from each well. Change the tip after each sample.

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

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

12 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 1,800 rpm for 2 minutes.
   c Centrifuge the IMP plate to 280 xg for 1 minute.

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 appears clear.

15 Remove the adhesive seal from the IMP plate.

16 Transfer 15 µl of the clear supernatant from each well of the IMP plate to the corresponding well of the new MIDI plate labeled with the ALP barcode. Change the tip after each sample.

SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3' Ends* on page 93 immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Adenylate 3' Ends

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

Illumina-Supplied Consumables

- (Optional) A-Tailing Control (CTA) (1 tube per 48 reactions)
- A-Tailing Mix (ATL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB)

User-Supplied Consumables

- Microseal 'B' Adhesive Seal
- RNase/DNase-free Reagent Reservoirs
- RNase/DNase-free Strip Tubes and Caps

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - A-Tailing Control or Resuspension Buffer
  - A-Tailing Mix

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

- Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up IMP* on page 90 and let stand to thaw at room temperature.
  - Briefly centrifuge the thawed ALP plate to 280 xg for 1 minute
  - Remove the adhesive seal from the ALP plate.
- Pre-heat the microheating system to 37°C.

Add ATL

1 Do one of the following:
- If using the in-line control reagent:
  - Briefly centrifuge the thawed A-Tailing Control tube to 600 xg for 5 seconds.
  - Dilute the A-Tailing Control to 1/100 in Resuspension Buffer (1 µl A-Tailing Control + 99 µl Resuspension Buffer) before use. Discard the diluted A-Tailing Control after use.
  - Add add 2.5 µl of diluted A-Tailing Control to each well of the ALP plate.
- If not using the in-line control reagent, add 2.5 µl of Resuspension Buffer to each well of the ALP plate.

2 Add 12.5 µl of thawed A-Tailing Mix to each well of the ALP plate. Mix thoroughly as follows:
a Seal the ALP plate with a Microseal 'B' adhesive seal.
b Shake the ALP plate on a microplate shaker at 1,800 rpm for 2 minutes.
c Centrifuge the ALP plate to 280 xg for 1 minute.

Incubate 1 ALP

1 Incubate the ALP plate on the pre-heated microheating system, with the lid closed, for 30 minutes at 37°C.

2 Immediately remove the ALP plate from the microheating system, then proceed immediately to *Ligate Adapters* on page 95.

Ligate Adapters

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

Illumina-Supplied Consumables

- Ligation Mix (LIG) (1 tube per 48 reactions)
- (Optional) Ligation Control (CTL) (1 tube per 48 reactions)
- Resuspension Buffer (RSB)
- RNA Adapter Indexes (AR001–AR016, AR018–AR023, AR025, AR027) (1 tube per column of 8 reactions, depending on the RNA Adapter Indexes being used)
- Stop Ligation Buffer (STL) (1 tube per 48 reactions)
- CAP (Clean Up ALP Plate) barcode label
- PCR (Polymerase Chain Reaction) barcode label

User-Supplied Consumables

- 96-well MIDI plate
- 96-well HSP plate
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'B' Adhesive Seals
- RNase/DNase-free Reagent Reservoirs
- RNase/DNase-free Strip Tubes and Caps

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - Appropriate RNA Adapter Index tubes, depending on the RNA Adapter Indexes being used
  - Stop Ligation Buffer
  - Ligation Control or Resuspension Buffer

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

- Review *AMPure XP Handling* on page 10.
- 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. Each column should contain a common index. This will facilitate pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.

Add LIG

1. Briefly centrifuge the appropriate/desired thawed RNA Adapter Index tubes, Ligation Control (if using Ligation Control), and Stop Ligation Buffer tubes to 600 xg for 5 seconds.
2. Immediately before use, remove the Ligation Mix tube from -15° to -25°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 add 2.5 µl of diluted Ligation Control to each well of the ALP plate.
   - If not using the in-line control reagent, add 2.5 µl of Resuspension Buffer to each well of the ALP plate.
5. Add 2.5 µl of Ligation Mix to each well of the ALP plate.
6. Return the Ligation Mix tube back to -15° to -25°C storage immediately after use.

7 Add 2.5 µl of the appropriate/desired 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 1,800 rpm for 2 minutes.
   c Centrifuge the ALP plate to 280 xg for 1 minute.

Incubate 2 ALP

1 Incubate the ALP plate on the pre-heated microheating system, with the lid closed, at 30°C for 10 minutes.

2 Remove the ALP plate from the microheating system.

Add STL

1 Remove the adhesive seal from the ALP plate.

2 Add 5 µl of Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation mix. Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1,800 rpm for 2 minutes.
   c Centrifuge the ALP plate to 280 xg for 1 minute.

Clean Up ALP

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 when working with AMPure XP Beads.

1 Remove the adhesive seal from the ALP plate.

2 Vortex the AMPure XP Beads until they are well dispersed, then add 42 µl of mixed AMPure XP Beads to each well of the ALP plate. Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1,800 rpm for 2 minutes.

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

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

5 Remove the adhesive seal from the ALP plate.

6 Remove and discard 79.5 µl of the supernatant from each well of the ALP plate. Change the tip after each sample.

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

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

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

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

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

11 Resuspend the dried pellet in each well with 52.5 µl Resuspension Buffer. Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1,800 rpm for 2 minutes.

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

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

14 Remove the adhesive seal from the ALP plate.

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

16 Vortex the AMPure XP Beads until they are well dispersed, then add 50 µl of mixed AMPure XP Beads to each well of the CAP plate. Mix thoroughly as follows:
   a Seal the CAP plate with a Microseal 'B' adhesive seal.
   b Shake the CAP plate on a microplate shaker at 1,800 rpm for 2 minutes.

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

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

19 Remove the adhesive seal from the CAP plate.

20 Remove and discard 95 µl of the supernatant from each well of the CAP plate. Take care not to disturb the beads. Change the tip after each sample.

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

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

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

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

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

25 Resuspend the dried pellet in each well with 22.5 µl Resuspension Buffer. Mix thoroughly as follows:

a Seal the CAP plate with a Microseal 'B' adhesive seal.

b Shake the CAP plate on a microplate shaker at 1,800 rpm for 2 minutes.

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

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

28 Remove the adhesive seal from the CAP plate.

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

SAFE STOPPING POINT
If you do not plan to proceed to *Enrich DNA Fragments* on page 100 immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR or SSP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Enrich DNA Fragments

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

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

Illumina-Supplied Consumables

- PCR Master Mix (PMM) (1 tube per 48 reactions)
- PCR Primer Cocktail (PPC) (1 tube per 48 reactions)
- Resuspension Buffer (RSB)
- CPP (Clean Up PCR Plate) barcode label
- TSP1 (Target Sample Plate) barcode label

User-Supplied Consumables

- 96-well MIDI plate
- 96-well HSP plate
- AMPure XP Beads
- Freshly Prepared 80% Ethanol (EtOH)
- Microseal 'A' Film
- Microseal 'B' Adhesive Seals
- RNase/DNase-free Reagent Reservoirs
- RNase/DNase-free Strip Tubes and Caps

Preparation

- Remove one tube each of PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature. Once thawed, keep the tubes on

ice.

- Briefly centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- Review *AMPure XP Handling* on page 10.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the PCR plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 97 and let stand to thaw at room temperature.
  - Briefly centrifuge the thawed PCR plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed PCR plate.
- Pre-program the thermal cycler with the following program and save as **PCR**:
  - 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
- Pre-heat the thermal cycler lid to 100°C.
- Apply a CPP barcode label to a new 96-well MIDI plate.
- Apply a TSP1 barcode label to a new 96-well HSP plate.

Make PCR

1. Add 5 µl of thawed PCR Primer Cocktail to each well of the PCR plate. Change the tip after each sample.
2. Add 25 µl of thawed PCR Master Mix to each well of the PCR plate. Change the tip after each sample. Mix thoroughly as follows:
   a. Seal the PCR plate with a Microseal 'A' film.
   b. Shake the PCR plate on a microplate shaker at 1,600 rpm for 20 seconds.
   c. Centrifuge the PCR plate to 280 xg for 1 minute.

WARNING

Read and follow the vendor's instructions for applying Microseal "A" sealing films. Improper use could lead to inefficient sealing (evaporation of sample or cross contamination) or too efficient sealing (parts of the seal remain in

the well after removing the whole seal).

Amp PCR

1. Pre-heat the thermal cycler lid to 100°C.
2. Amplify the PCR plate in the pre-programmed thermal cycler, with the lid closed, as follows:
   a. 98°C for 30 seconds
   b. 15 cycles of:
      98°C for 10 seconds
      60°C for 30 seconds
      72°C for 30 seconds
   c. 72°C for 5 minutes
   d. Hold at 10°C

Clean Up PCR

NOTE
Before performing clean up, review *AMPure XP Handling* on page 10 when working with AMPure XP Beads.

1. Remove the adhesive seal from the PCR plate.
2. Vortex the AMPure XP Beads until they are well dispersed, then add 50 µl of the mixed AMPure XP Beads to each well of the new MIDI plate labeled with the CPP barcode. Change the tip after each sample.
3. Transfer the entire contents from each well of the PCR plate to the corresponding well of the CPP plate containing 50 µl of mixed AMPure XP Beads. Mix thoroughly as follows:
   a. Seal the CPP plate with a Microseal 'B' adhesive seal.
   b. Shake the CPP plate on a microplate shaker at 1,800 rpm for 2 minutes.
4. Incubate the CPP plate at room temperature for 15 minutes.
5. Place the CPP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.
6. Remove the adhesive seal from the CPP plate.

7 Remove and discard 95 µl of the supernatant from each well of the CPP plate. Take care not to disturb the beads. Change the tip after each sample.

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

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

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

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

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

12 Resuspend the dried pellet in each well with 32.5 µl Resuspension Buffer. Mix thoroughly as follows:
   a Seal the CPP plate with a Microseal 'B' adhesive seal.
   b Shake the CPP plate on a microplate shaker at 1,800 rpm for 2 minutes.

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

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

15 Remove the adhesive seal from the CPP plate.

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

SAFE STOPPING POINT
If you do not plan to proceed to *Validate Library* on page 104 immediately, the protocol can be safely stopped here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Validate Library

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

Quantify Libraries

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

Quality Control

1. Load 1 µl of the resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA specific chip such the Agilent DNA-1000.
2. Check the size and purity of the sample. The final product should be a band at approximately 260 bp (for single-read libraries).

Figure 19 Example of TruSeq RNA Sample Prep v2 Library Size Distribution

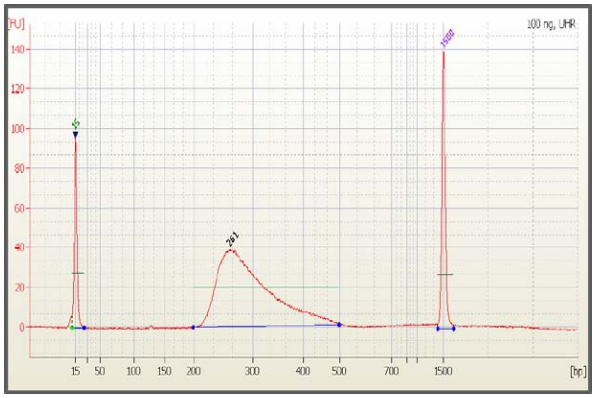
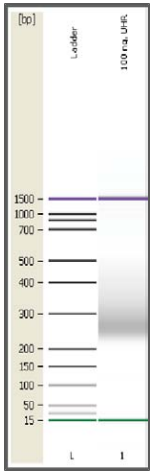


Figure 20 TruSeq RNA Sample Prep v2 260 bp PCR Product



Normalize and Pool Libraries (Optional)

This process describes how to prepare DNA templates that will be applied to cluster generation. Multiplexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. Non-multiplexed DNA libraries are normalized to 10 nM in the DCT plate without pooling.

Illumina-Supplied Consumables

- DCT (Diluted Cluster Template) barcode label
- PDP (Pooled DCT Plate) barcode label (for multiplexing only)

User-Supplied Consumables

- 96-well HSP plate (for multiplexing only)
- 96-well MIDI plate
- Microseal 'B' Adhesive seals
- Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20

Preparation

- Apply a DCT barcode label to a new 96-well MIDI plate.
- Apply a PDP barcode label to a new 96-well HSP plate (for multiplexing only).
- Remove the TSP1 plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 102, and let stand to thaw at room temperature.
  - Briefly centrifuge the thawed TSP1 plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.

Make DCT

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

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

3 Mix the DCT plate as follows:
   a Seal the DCT plate with a Microseal 'B' adhesive seal.
   b Shake the DCT plate on a microplate shaker at 1,000 rpm for 2 minutes.
   c Centrifuge the DCT plate to 280 xg for 1 minute.
   d Remove the adhesive seal from the DCT plate.

4 Depending on the type of library you want to generate, do one of the following:
   - For non-multiplexed paired-end libraries, the protocol stops here. Do one of the following:
     — Proceed to cluster generation. See the *Illumina Cluster Generation User Guide*.
     — Seal the DCT plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.
   - For multiplexed paired-end libraries, proceed to Make PDP.

Make PDP (for multiplexing 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 should be 10X the number of combined sample libraries and will be 10–120 µl (1–12 libraries).

Table 12 Pooled Sample Volumes

Number of pooled samples	Volume (µl)
1	10
2	20

Number of pooled samples	Volume (µl)
3	30
4	40
5	50
6	60
7	70
8	80
9	90
10	100
11	110
12	120

NOTE
Keep track of which sample goes into which well, to 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 1,800 rpm for 2 minutes.

4 Do one of the following:
   - Proceed to cluster generation. See the *Illumina Cluster Generation User Guide*.
   - Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.

Alternate Fragmentation Protocols

Appendix A

Introduction

Fragmentation of the nucleic acids is required for optimal library preparation, clustering and sequencing. The fragmentation protocol for transcriptome analysis is performed on the RNA after mRNA purification using elevated temperatures. The fragmentation protocol included in this guide results in libraries with inserts ranging in size from 120-200 bp with a median size of 150 bp. This 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. This can be accomplished during the Purify and Fragment mRNA procedures by modifying the thermal cycler **Elution 2 - Frag - Prime** program: 94°C for X minutes followed by a 4°C hold for the thermal cycler where X is determined by the length of RNA desired. A range of suggested times and sizes is described in .

Table 13 Library Insert Fragmentation Time

Time at 94 °C (minutes)	Range of Insert Length[a] (bp)	Median Insert Length[a] (bp)
0	130—350	200
1	130—310	190
2	130—290	185
3	125—250	165
4	120—225	160
8	120—210	155
12	115—180	140
Covaris[b]	130—280	180

a.Insert length determined after clustering and sequencing with a paired-end sequencing run.
b.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.

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 76° to 80°C for 2 minutes followed by a 4°C hold. This will elute the mRNA and anneal the random primers without fragmenting the RNA. Proceed with the protocol through the Clean Up CDP procedures to purify the ds cDNA. At this point, 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° to 6°C. You man 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:
   - Duty cycle—5%
   - Intensity—3
   - Bursts per second—200
   - Duration—80 seconds
   - Mode—Frequency sweeping
   - Power—33–34W
   - Temperature—5.5° to 6°C

3 Seal the Covaris tube and briefly centrifuge to 600 xg for 5 seconds.

4 Transfer contents to IMP plate and proceed to *Perform End Repair*.

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Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 14 Illumina General Contact Information

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

Table 15 Illumina Customer Support Telephone Numbers

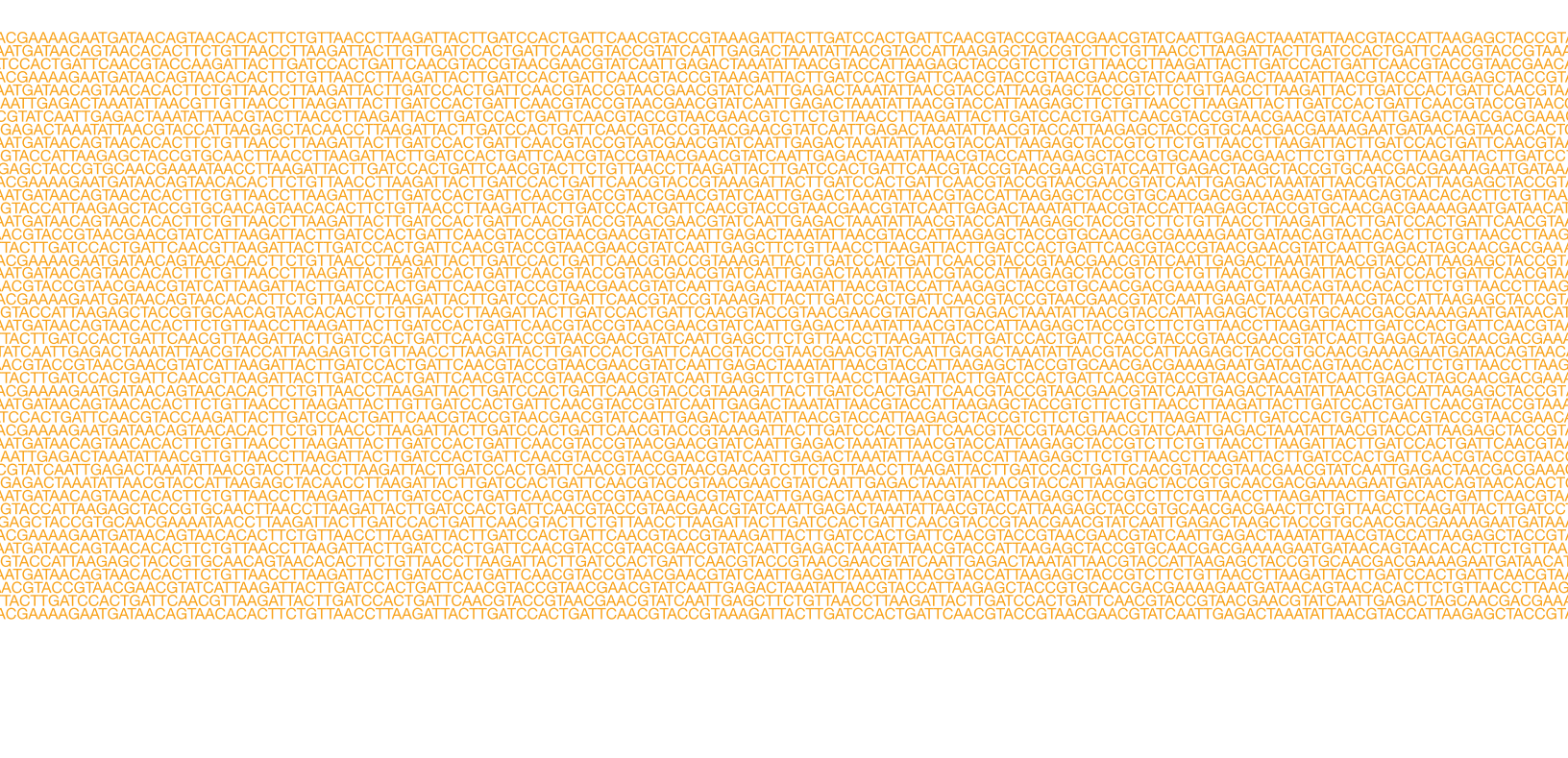
Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994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

MSDSs

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

Product Documentation

If you require additional product documentation, you can obtain PDFs from the Illumina website if PDFs are available. Go to http://www.illumina.com/support/documentation.ilmn. When you click on a link, you will be asked to log in to iCom. After you log in, you can view or save the PDF. To register for an iCom account, please visit https://icom.illumina.com/Account/Register.



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