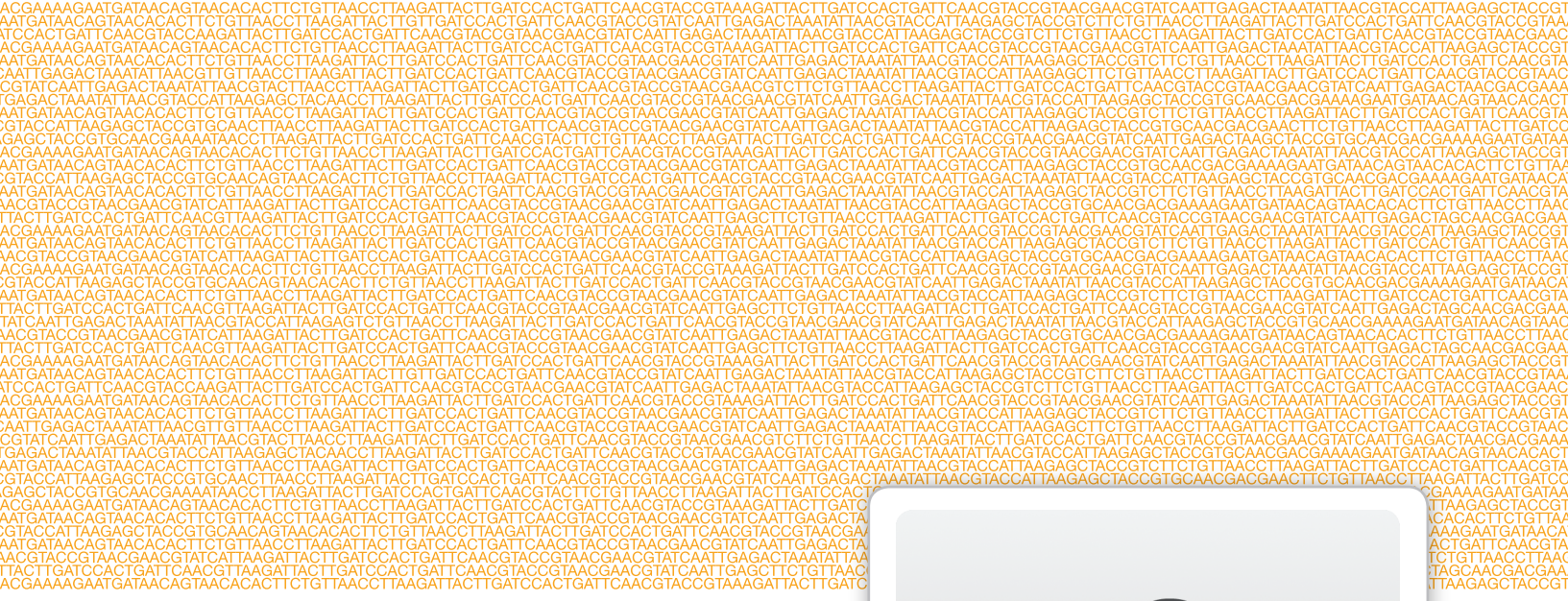




TruSeq™ RNA Sample Preparation Guide



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Revision History

Part #	Revision	Date	Description of Change
15008136	A	November 2010	Initial Release

Revision History

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Introduction

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

- ▶ Chapter 3, explains how to perform TruSeq RNA Sample Preparation using the *TruSeq RNA Sample Preparation Low Throughput (LT) Protocol*
- ▶ Chapter 4, explains how to perform TruSeq RNA Sample Preparation using the *TruSeq RNA Sample Preparation 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 TCY
Incubation Equipment	96-well thermal cycler	96-well thermal cycler Microheating system
Mixing Method	Pipetting	Micro plate shaker

Standard Operating Procedures

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Introduction

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

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

Acronyms

Table 2 TruSeq RNA Sample Preparation 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	Complementary DNA
CDP	cDNA Plate
CPP	Clean Up PCR Plate
CTA	A-Tailing Control
CTE	End Repair Control
CTL	Ligase 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

Table 2 TruSeq RNA Sample Preparation Acronyms (Continued)

Acronym	Definition
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	RNA Fragmentation Plate
RPB	RNA Purification Beads
RSB	Resuspension Buffer
SSM	Second Strand Master Mix
STL	Stop Ligase Mix
TCY	Skirted Thermocycler Plate
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 (ds) DNA.
- ▶ 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 ($\pm 0.5 \mu\text{l}$) can sometimes give rise to very large differences in cluster numbers ($\sim 100,000$).
- ▶ 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, because solutions of high molecular weight dsDNA can be viscous and not evenly dispersed, resulting in aliquot measurements that are not representative of the true concentration of the solution.

AMPure XP Handling

The following indicates the appropriate handling methods when working with Agencourt AMPure XP Beads:

- ▶ 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 thoroughly by pipetting up and down 10 times.
- ▶ **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.
- ▶ Change the tips for each sample or when using a multichannel pipette, change the tips after each column.
- ▶ Let the mixed samples incubate for 15 minutes at room temperature for maximum recovery.
- ▶ The minimum time required for the beads to be separated from the solution is 5 minutes, but may be longer depending on the volume of the reaction and on the magnetic stand used. Wait at least 5 minutes for the solution to clear before proceeding to the next step.
- ▶ 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 μ l of supernatant are left when the eluates are removed from the bead pellet.
- ▶ For the wash steps, prepare fresh 80% ethanol. Eighty-percent 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.

- ▶ Remove the reaction plate from the magnetic stand and let it air-dry at room temperature. Allow for the complete evaporation of residual ethanol, as it impacts the performance of the subsequent reactions. If the beads are not completely dried, it is also difficult to remove the eluted DNA in the supernatant without significant bead contamination. Illumina recommends at least 15 minutes drying time, but a longer drying time may be required.
- ▶ Use the Resuspension Buffer for DNA elution.
 - **When performing the LT protocol**, gently pipette 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**, seal the plate and shake the plate on a microplate shaker at 1,800 rpm for 2 minutes,
- ▶ 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

- ▶ 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.

Temperature Considerations

Temperature is another important consideration for making cDNA libraries. Elevated temperatures should be particularly avoided 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. As a general rule, libraries should be kept at temperatures $\leq 37^{\circ}\text{C}$. Temperature is less of an issue after the adapters have been ligated onto the ends of the double-stranded cDNA.

RNA Input Recommendations

Total RNA Input

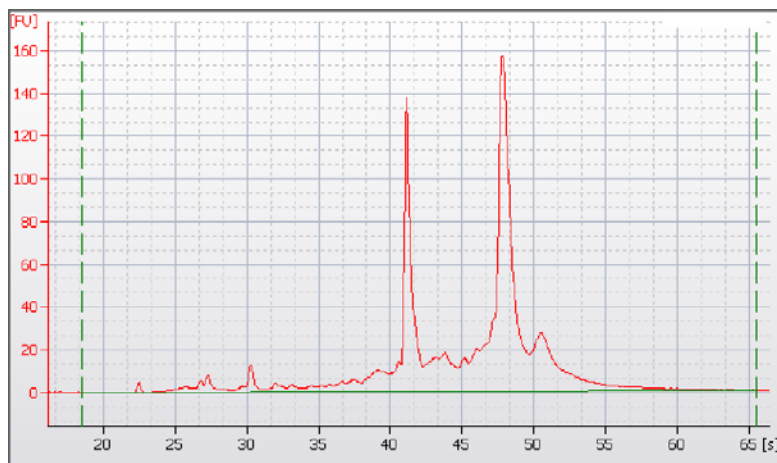
This protocol is optimized for 0.1–4 µg of total RNA. Lower amounts may result in inefficient ligation and low yield. The protocol has been tested using 0.1–10 µg of high-quality universal human reference total RNA as input. Use of RNA from other species, tissues, or qualities may require further optimization 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 RSB 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 5' 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 1 Starting RNA Bioanalyzer Trace



Alternatively, you can run a formaldehyde 1% agarose gel and judge the integrity of RNA upon staining with ethidium bromide. High quality RNA shows a 28S rRNA band at 4.5 kb 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 step 11 of the *Make RFP* procedures.

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 Ligase Control reagents contain DNA fragments used as controls for the enzymatic activities of the End Repair Mix, A-Tailing Mix, and DNA Ligase 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 a control's sequence 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, each controls yields approximately 0.1% 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
DNA Ligase Mix	Ligation: Join adapters to inserts	Ligase 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 1.8 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 Form** 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. 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.

Figure 2 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	i. doe

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
Sample Ref	The reference sequence for the sample
Index	The index sequence or 0 for no indexing
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



CAUTION

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

Kit Contents

Check to ensure that you have all of the reagents identified in this section before proceeding. Each TruSeq RNA Sample Preparation Kit can be used to process up to 48 samples. The kit carton also contains plate barcode labels.

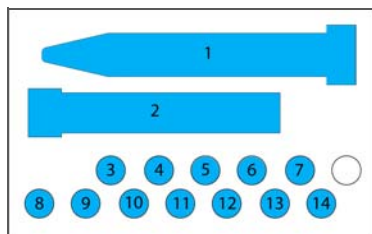
Kit Contents, Boxes A and B

You will choose to receive either box A or B with the kit depending up which indices you require.

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.

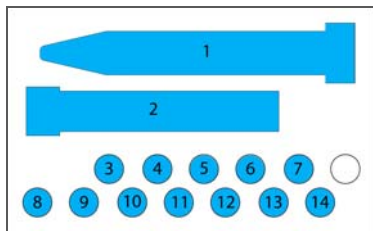
Figure 3 TruSeq RNA Sample Preparation Kit, Box A, part # 15013135



- 1 Resuspension Buffer (RSB), part # 15012547
- 2 End Repair Mix (ERP), part # 15012494
- 3 A-Tailing Mix (ATL), part # 15012495
- 4 Ligation Mix (LIG), part # 15012496
- 5 End Repair Control (CTE), part # 15012360
- 6 A-Tailing Control (CTA), part # 15012361
- 7 Ligase Control (CTL), part # 15012362
- 8 Stop Ligation Buffer (STL), part # 15012546
- 9 RNA Adapter Index 2 (AR002), part # 15013689
- 10 RNA Adapter Index 4 (AR004), part # 15013691

- 11 RNA Adapter Index 5 (AR005), part # 15013692
- 12 RNA Adapter Index 6 (AR006), part # 15013693
- 13 RNA Adapter Index 7 (AR007), part # 15013694
- 14 RNA Adapter Index 12 (AR012), part # 15013699

Figure 4 TruSeq RNA Sample Preparation Kit, Box B, part # 15013136



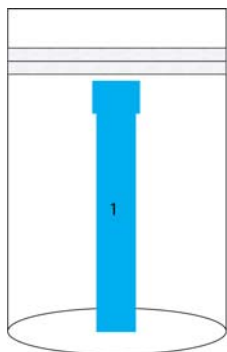
- 1 Resuspension Buffer (RSB), part # 15012547
- 2 End Repair Mix (ERP), part # 15012494
- 3 A-Tailing Mix (ATL), part # 15012495
- 4 Ligation Mix (LIG), part # 15012496
- 5 End Repair Control (CTE), part # 15012360
- 6 A-Tailing Control (CTA), part # 15012361
- 7 Ligase Control (CTL), part # 15012362
- 8 Stop Ligation Buffer (STL), part # 15012546
- 9 RNA Adapter Index 1 (AR001), part # 15013688
- 10 RNA Adapter Index 3 (AR003), part # 15013690
- 11 RNA Adapter Index 8 (AR008), part # 15013695
- 12 RNA Adapter Index 9 (AR009), part # 15013696
- 13 RNA Adapter Index 10 (AR010), part # 15013697
- 14 RNA Adapter Index 11 (AR011), part # 15013698

Kit Contents, Box 1

Store at 2° to 8°C

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

Figure 5 TruSeq RNA Sample Preparation Kit, Box 1, part # 15012996



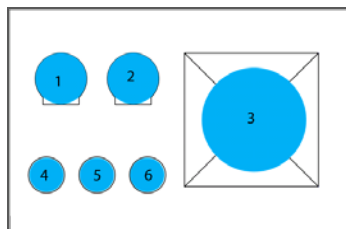
- 1 RNA Purification Beads (RPB), part # 15012932

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 6 TruSeq RNA Sample Preparation Kit Box 2, part # 15012997



- 1 Bead Binding Buffer (BBB), part # 15012927
- 2 Elution Buffer (ELB), part # 15012900

- 3 Bead Washing Buffer (BWB), part # 15012925
- 4 Elute, Prime, Fragment Mix (EPF), part # 15016648
- 5 First Strand Master Mix (FSM), part # 15016647
- 6 Second Strand Master Mix (SSM), part # 15012909

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 7 TruSeq RNA Sample Preparation Kit, PCR Prep Box, part # 15012995

		1		
		2		

- 1 PCR Master Mix (PMM), part # 15013681
- 2 PCR Primer Cocktail (PPC), part # 15013682

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 protocols. The requirement of some supplies are dependant upon the exact protocol followed (LT or HT) and these items are specified in separate tables below.

Table 5 User-Supplied Consumables

Consumable	Supplier
1.5 ml RNase/DNase-free non-sticky tubes	Ambion, part # AM12450
10 µl multichannel pipettes	General lab supplier
10 µl barrier pipette tips	General lab supplier
10 µl single channel pipettes	General lab supplier
100% Ethanol	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl single channel pipettes	General lab supplier
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
RNase/DNase zapper (to decontaminate surfaces)	General lab supplier
Freshly Prepared 80% Ethanol	General lab supplier
Microseal 'B' Adhesive seals	BioRad, part # MSB1001
RNase/DNase-free Multichannel reagent reservoirs, disposable	General lab supplier
RNase/DNase-free Strip tubes and caps	General lab supplier
SuperScript II Reverse Transcriptase	Invitrogen, part # 18064-014

Table 5 User-Supplied Consumables (Continued)

Consumable	Supplier
Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20, or QIAGEN EB Buffer	General lab supplier, or QIAGEN, part # 19086
Tween 20	Sigma, part # P7949
Ultra pure water	General lab supplier

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

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

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

Consumable	Supplier
96-well storage plates, round well, 0.8 ml ("MIDI" plate)	Fisher Scientific, part # AB-0859
Microseal 96-well PCR plates ("TCY" plate)	BIO-RAD, part # HSP-9601

Table 8 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 9 User-Supplied Equipment - Additional Items for HT Processing

Equipment	Supplier
High Speed Micro Plate Shaker	Illumina, part # 175732
MIDI plate insert for heating system	Illumina, part # 211191
Stroboscope (to calibrate the micro plate shaker)	General lab supplier
Tru Temp Microheating System	Illumina, part # 11191471

Low-Throughput (LT) Protocol

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Introduction

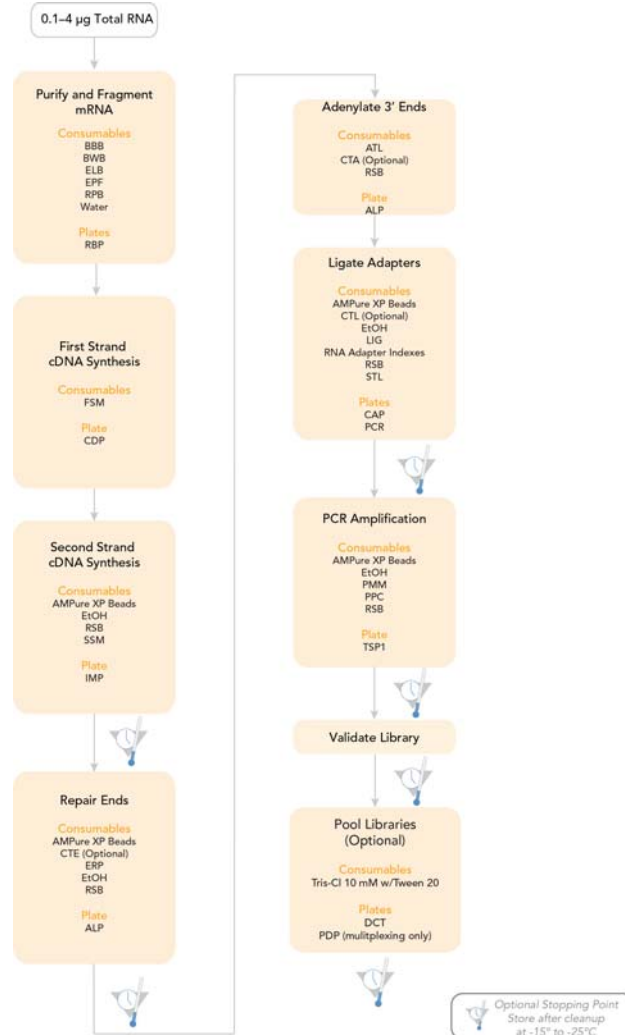
This chapter describes the TruSeq RNA Sample Preparation low-throughput (LT) protocol. Illumina recommends this protocol for processing 48 or fewer samples. Follow the protocol in the order shown. For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample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

When processing more than 48 samples, Illumina recommends following the *High-Throughput (HT) Protocol*.

Sample Prep Workflow

The following illustrates the processes of the LT TruSeq RNA Sample Preparation protocol to prepare a template composed of 12 pooled indexed libraries.

Figure 8 TruSeq RNA Sample Preparation LT Workflow

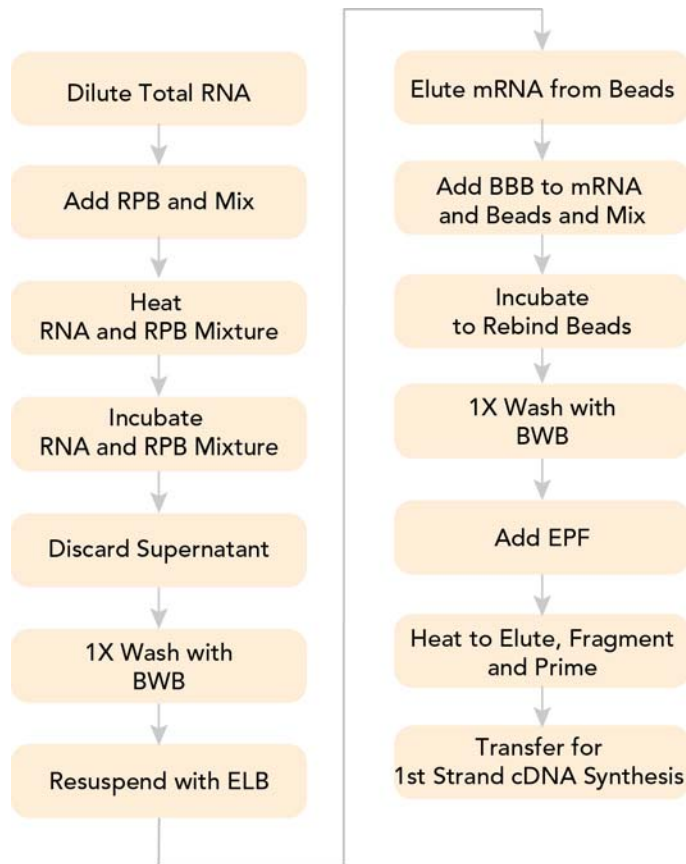


Purify and Fragment mRNA

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 9 TruSeq RNA Purification Workflow



It is important to follow this procedure exactly to ensure reproducibility.



NOTE

Allow the beads to fully pellet against the magnetic stand for at least 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* in this process.



NOTE

For inserts larger than 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)
- ▶ Bead Washing Buffer (BWB) (1 tube)
- ▶ Elution Buffer (ELB) (1 tube)
- ▶ Elute, Prime, Fragment Mix (EPF) (1 tube)
- ▶ RNA Purification Beads (RPB) (1 tube)
- ▶ 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
- ▶ RNase/DNase-free Strip Tubes and Caps
- ▶ Ultra Pure Water

Preparation

- ▶ For first time use, remove one tube each of Bead Binding Buffer, Bead Washing Buffer, Elution Buffer, and Elute, Prime, Fragment Mix from -15° to -25°C storage and thaw them at room temperature.
- ▶ 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

When using a multichannel pipette:

- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.



NOTE

After use in this procedure, store the Bead Binding Buffer, Bead Washing Buffer, and Elution Buffer store 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 with the RBP barcode label.
- 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 using a multichannel pipette 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. Change the tips after each column.

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

Incubate 1 RBP

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

Wash RBP

- 1 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 using a multichannel pipette. Take care not to disturb the beads. Change the tips after each column.
- 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 using a multichannel pipette to remove unbound RNA. Gently pipette the entire volume up and down 6 times to mix thoroughly. Change the tips after each column.
- 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 using a multichannel pipette. Take care not to disturb the beads. Change the tips after each column.
- 9 Remove the RBP plate from the magnetic stand.
- 10 Add 50 µl of Elution Buffer in each well of the RBP plate using a multichannel pipette. Gently pipette the entire volume up and down 6 times to mix thoroughly. Change the tips after each column.
- 11 Seal the RBP plate with a Microseal 'B' Adhesive seal.

- 12 Store the Elution Buffer tube at 4°C.

Incubate 2 RBP

- 1 Place the sealed RBP plate on the pre-programmed thermal cycler. Close the lid and select **mRNA Elution 1** (80°C for 2 minutes, 25°C hold) to elute the mRNA from the beads.
- 2 Remove the RBP plate from the thermal cycler when it reaches 25°C.
- 3 Place the RBP plate on the bench at room temperature 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 using a multichannel pipette to allow the RNA to re-bind to the beads. Gently pipette the entire volume up and down 6 times to mix thoroughly. Change the tips after each column.
- 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 using a multichannel pipette. Take care not to disturb the beads. Change the tips after each column.
- 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 using a multichannel pipette. Gently pipette the entire volume up and down 6 times to mix thoroughly. Change the tips after each column.
- 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 using a multichannel pipette. Take care not to disturb the beads. Change the tips after each column.
- 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 using a multichannel pipette. Gently pipette the entire volume up and down 6 times to mix

thoroughly. Change the tips after each column. 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 Place the sealed RBP plate on the pre-programmed thermal cycler. Close the lid and select **Elution 2 - Frag - Prime** (94°C for 8 minutes, 4°C hold) to elute, fragment, and prime the RNA.
- 2 Remove the RBP plate from the thermal cycler when it reaches 4°C and centrifuge briefly.
- 3 Proceed immediately to *Synthesize First Strand cDNA*.

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
- ▶ 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.
- ▶ 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. 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.



NOTE

When using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C .
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

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. Some liquid may remain in each well.
- 4 Briefly centrifuge the thawed First Strand Master Mix 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 7 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 using a multichannel pipette. Gently pipette the entire volume up and down 6 times to mix thoroughly. Change the tips after each column.
- 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 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
- 2 When the thermal cycler reaches 4°C, remove the CDP plate from the thermal cycler and proceed immediately to *Synthesize Second Strand cDNA*.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand to generate double-stranded (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
- ▶ 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.
- ▶ 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.
- ▶ Review *AMPure XP Handling* on page 20.

**NOTE**

When using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

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 μ l of thawed Second Strand Master Mix to each well of the CDP plate using a multichannel pipette. Gently pipette the entire volume up and down 6 times to mix thoroughly. Change the tips after each column.
- 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 20 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 at least 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 using a multichannel pipette. Some liquid may remain in each well. Take care not to disturb the beads. Change the tips after each column.



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 using a multichannel pipette. Take care not to disturb the beads. Change the tips after each column.
- 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 $\times g$ for 5 seconds.
- 10 Add 52.5 μl Resuspension Buffer to each well of the CDP plate using a multichannel pipette. 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. Some liquid may remain in each well.



SAFE STOPPING POINT

If you do not plan to proceed to *Perform End Repair* immediately, the protocol can be safely stopped here. If you are stopping, seal the IMP plate with a Microseal 'B' adhesive seal and store it 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 (ERP) 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
- ▶ RNase/DNase-free Strip Tubes and Caps

Preparation

- ▶ Remove one tube of End Repair Mix and, if using End Repair Control, one tube of End Repair Control per 48 reactions from -15° to -25°C storage and thaw them at room temperature.



NOTE

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



NOTE

If you do not intend to consume the End Repair Control and End Repair Mix reagents in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- ▶ Remove the IMP plate from -15° to -25°C storage if it was stored at the conclusion of *Synthesize Second Strand cDNA* and let stand to thaw at room temperature.
- ▶ Review *AMPure XP Handling* on page 20.
- ▶ 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.



NOTE

When using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C .
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Make IMP

- 1 If using the in-line control End Repair Control, briefly centrifuge the thawed End Repair Control tube to $600 \times g$ for 5 seconds and dilute the End Repair Control to 1/100 in Resuspension Buffer ($1 \mu\text{l}$ End Repair Control + $99 \mu\text{l}$ Resuspension Buffer) before use. Discard the diluted End Repair Control after use.
- 2 Add $10 \mu\text{l}$ of diluted End Repair Control (or $10 \mu\text{l}$ of Resuspension Buffer if not using End Repair Control) to each well of the IMP plate that contains $50 \mu\text{l}$ of ds cDNA using a multichannel pipette. Change the tips after each column.
- 3 Add $40 \mu\text{l}$ of End Repair Mix to each well of the IMP plate containing the ds cDNA and End Repair Control using a multichannel pipette. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 4 Seal the IMP plate with a Microseal 'B' adhesive seal.

Incubate 1 IMP

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

Clean Up IMP



NOTE

Before performing clean up, review *AMPure XP Handling* on page 20 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 μ l of well-mixed AMPure XP Beads to each well of the IMP plate containing 100 μ l of End Repair Mix. Gently pipette up and down 10 times to mix thoroughly.
- 3 Incubate the IMP plate at room temperature for 15 minutes.
- 4 Place the IMP plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
- 5 Using a 200 μ l multichannel pipette set to 127.5 μ l, remove and discard 127.5 μ l of the supernatant from each well of the IMP plate. Take care not to disturb the beads. Change the tips after each column.
- 6 Repeat step 5 once. Some liquid may remain in each well.



NOTE

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

- 7 With the IMP plate on the magnetic stand, add 200 μ l of freshly prepared 80% EtOH to each well without disturbing the beads.
- 8 Incubate the IMP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tips after each column.
- 9 Repeat steps 7 and 8 once for a total of two 80% EtOH washes.

- 10 Let the IMP plate stand at room temperature for 15 minutes to dry and then remove the plate from the magnetic stand.
- 11 Resuspend the dried pellet in 17.5 μ l Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 12 Incubate the IMP plate at room temperature for 2 minutes.
- 13 Place the IMP plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
- 14 Transfer 15 μ l 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. Some liquid may remain in each well.



SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3' Ends* immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store it 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) (1 tube)

USER-SUPPLIED CONSUMABLES

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

Preparation

- ▶ Remove one tube of A-Tailing Mix and, if using A-Tailing Control, one tube of A-Tailing Control per 48 reactions from -15° to -25°C storage and thaw them at room temperature.



NOTE

If you do not intend to consume the A-Tailing Mix and A-Tailing Control reagents in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- ▶ Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Perform End Repair* and let stand to thaw at room temperature.
- ▶ Briefly centrifuge the thawed ALP plate to 280 xg for 1 minute, then remove the adhesive seal from the plate.
- ▶ Pre-heat the thermal cycler to 37°C.

**NOTE**

When using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Add ATL

- 1 If using the in-line control A-Tailing Control, briefly centrifuge the thawed A-Tailing Control tube to 600 xg for 5 seconds and dilute the A-Tailing Control to 1/100 in Resuspension Buffer (1 μ l A-Tailing Control + 99 μ l Resuspension Buffer) before use. Discard the diluted A-Tailing Control after use.
- 2 Add 2.5 μ l of diluted A-Tailing Control (or 2.5 μ l of Resuspension Buffer if not using A-Tailing Control) to each well of the ALP plate using a multichannel pipette.
- 3 Adjust the multichannel pipette to 30 μ l and gently pipette the entire volume up and down 10 times to mix thoroughly.
- 4 Add 12.5 μ l of thawed A-Tailing Mix to each well of the ALP plate using a multichannel pipette. Change the tips after each column.
- 5 Seal the ALP plate with a Microseal 'B' adhesive seal.

Incubate 1 ALP

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

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

- ▶ DNA Ligase Mix (LIG) (1 tube per 48 reactions)
- ▶ (Optional) Ligase Control (CTL) (1 tube per 48 reactions)
- ▶ Resuspension Buffer (RSB) (1 tube)
- ▶ RNA Adapter Indexes 1–12 (AR001–AR012) (1 tube of each, depending on the RNA Adapter Indexes being used)
- ▶ Stop Ligase Mix (STL)
- ▶ 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
- ▶ RNase/DNase-free Strip Tubes and Caps

Preparation

- ▶ Remove the appropriate RNA Adapter Index tubes (AR001–AR012, depending on the RNA Adapter Indexes being used), and one tube Stop Ligase Mix and, if using Ligase Control, one tube of Ligase Control, per 48 reactions from -15° to -25°C storage and thaw them at room temperature.



NOTE

If you do not intend to consume the Ligase Control in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- ▶ Review *AMPure XP Handling* on page 20.

- ▶ 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 using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Add LIG

- 1 Briefly centrifuge the thawed RNA Adapter Index tubes (AR001–AR012 depending on the RNA Adapter Indexes being used), Ligase Control (if using Ligase Control), and Stop Ligase Mix tubes to 600 xg for 5 seconds.
- 2 Immediately before use, remove the DNA Ligase Mix tube from -15° to -25°C storage.

**NOTE**

If you do not intend to consume the DNA Ligase Mix in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- 3 Remove the adhesive seal from the ALP plate.
- 4 Add 2.5 µl of DNA Ligase Mix to each well of the ALP plate.
- 5 Return the DNA Ligase Mix tube back to -15° to -25°C storage immediately after use.
- 6 If using the in-line control Ligase Control, dilute the Ligase Control to 1/100 in Resuspension Buffer (1 µl Ligase Control + 99 µl Resuspension Buffer) before use. Discard the diluted Ligase Control after use.

- 7 Add 2.5 μ l of diluted Ligase Control (or 2.5 μ l of Resuspension Buffer if not using Ligase Control) to each well of the ALP plate.
- 8 Add 2.5 μ l of each thawed RNA Adapter Index (AR001–AR012 depending on the RNA Adapter Indexes being used) to each well of the ALP plate using a multichannel pipette.
- 9 Adjust the multichannel pipette to 40 μ l and gently pipette the entire volume up and down 10 times to mix thoroughly.
- 10 Seal the ALP plate with a Microseal 'B' adhesive seal.



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.

Incubate 2 ALP

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

Add STL

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

Clean Up ALP



NOTE

Before performing clean up, review *AMPure XP Handling* on page 20 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 using a multichannel pipette. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 2 Incubate the ALP plate at room temperature for 15 minutes.
- 3 Place the ALP plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
- 4 Remove and discard 79.5 μl of the supernatant from each well of the ALP plate using a multichannel pipette. Some liquid may remain in each well. Take care not to disturb the beads. Change the tips after each column.

**NOTE**

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

- 5 With the ALP plate remaining on the magnetic stand, add 200 μl of freshly prepared 80% EtOH to each well without disturbing the beads.
- 6 Incubate the ALP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tips after each column.
- 7 Repeat steps 5 and 6 once for a total of two 80% EtOH washes.
- 8 Let the ALP plate stand at room temperature for 15 minutes to dry and then remove the plate from the magnetic stand.
- 9 Resuspend the dried pellet in each well with 52.5 μl Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 10 Incubate the ALP plate at room temperature for 2 minutes.
- 11 Place the ALP plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
- 12 Transfer 50 μl of the clear supernatant from each well of the ALP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CAP barcode. Some liquid may remain in each well. Change the tips after each column.
- 13 Vortex the AMPure XP Beads until they are well dispersed, then add 50 μl of mixed AMPure XP Beads to each well of the CAP plate for a second clean up using a multichannel pipette. Gently pipette the entire volume up and down 10 times to mix thoroughly.

- 14 Incubate the CAP plate at room temperature for 15 minutes.
- 15 Place the CAP plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.
- 16 Remove and discard 95 μ l of the supernatant from each well of the CAP plate, using a multichannel pipette. Some liquid may remain in each well. Take care not to disturb the beads. Change the tips after each column.



NOTE

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

- 17 With the CAP plate remaining on the magnetic stand, add 200 μ l of freshly prepared 80% EtOH to each well without disturbing the beads.
- 18 Incubate the CAP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tips after each column.
- 19 Repeat steps 17 and 18 once for a total of two 80% EtOH washes.
- 20 Let the CAP plate stand at room temperature for 15 minutes to dry and then remove the plate from the magnetic stand.
- 21 Resuspend the dried pellet in each well with 22.5 μ l Resuspension Buffer. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 22 Incubate the CAP plate at room temperature for 2 minutes.
- 23 Place the CAP plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
- 24 Transfer 20 μ l of the clear supernatant from each well of the CAP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the PCR barcode. Some liquid may remain in each well. Change the tips after each column.



SAFE STOPPING POINT

If you do not plan to proceed to *Enrich DNA Fragments* immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store it 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)
- ▶ PCR Primer Cocktail (PPC) (1 tube)
- ▶ Resuspension Buffer (RSB) (1 tube)
- ▶ 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
- ▶ 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.
- ▶ Briefly centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- ▶ Review *AMPure XP Handling* on page 20.
- ▶ 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 *Ligate Adapters* 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 4°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.



NOTE

When using a multichannel pipette:

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Make PCR

- 1 Add 5 μl of thawed PCR Primer Cocktail to each well of the PCR plate using a multichannel pipette. Change the tips after each column.
- 2 Add 25 μl of thawed PCR Master Mix to each well of the PCR plate using a multichannel pipette. Change the tips after each column. Adjust the single channel or multichannel pipette to 40 μl and gently pipette the entire volume up and down 10 times to mix thoroughly.

Amp PCR

- 1 Amplify the PCR plate in the pre-programed thermal cycler, with the lid closed, using the **PCR** program:
 - 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 4°C

Clean Up PCR



NOTE

Before performing clean up, review *AMPure XP Handling* on page 20 when working with AMPure XP Beads.

- 1 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 using a multichannel pipette. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 2 Incubate the PCR plate at room temperature for 15 minutes.
- 3 Place the PCR plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.

- 4 Remove and discard 95 μl of the supernatant from each well of the PCR plate, using a multichannel pipette. Some liquid may remain in each well. Take care not to disturb the beads. Change the tips after each column.



NOTE

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

- 5 With the PCR plate remaining on the magnetic stand, add 200 μl of freshly prepared 80% EtOH to each well without disturbing the beads.
- 6 Incubate the PCR plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tips after each column.
- 7 Repeat steps 5 and 6 once for a total of two 80% EtOH washes.
- 8 Let the PCR plate stand at room temperature for 15 minutes to dry and then remove the plate from the magnetic stand.
- 9 Resuspend the dried pellet in each well with 32.5 μl Resuspension Buffer using a multichannel pipette. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 10 Incubate the PCR plate at room temperature for 2 minutes.
- 11 Place the PCR plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
- 12 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. Some liquid may remain in each well. Change the tips after each column.



SAFE STOPPING POINT

If you do not plan to proceed to *Validate Library* immediately, the protocol can be safely stopped here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store it 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 *qPCR Quantification Protocol Guide*.

Quality Control

- 1 Load 1 μ l 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 10 Example of TruSeq RNA Library Size Distribution

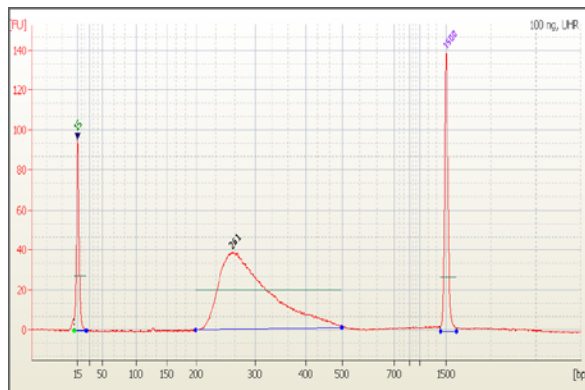
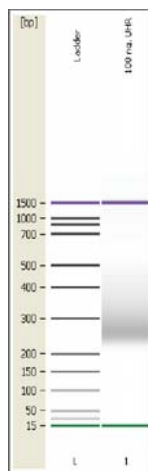


Figure 11 TruSeq RNA 260 bp PCR Product



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 (Diluted Cluster Template) plate and then pooled in equal volumes in the PDP (Pooled DCT 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 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:
 - a 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 it at -15° to -25°C .
 - b For multiplexed paired-end libraries, proceed to *Make PDP*.

Make PDP



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 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 10 Pooled Sample Volumes

Number of pooled samples	Volume (μl)
1	10
2	20
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 Gently pipette the entire volume up and down 10 times to mix thoroughly.
- 4 Do one of the following:
 - a Proceed to cluster generation. See the *Illumina Cluster Generation User Guide*.
 - b Seal the PDP plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C .

High-Throughput (HT) Protocol

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Introduction

This chapter describes the TruSeq RNA Sample Preparation 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.

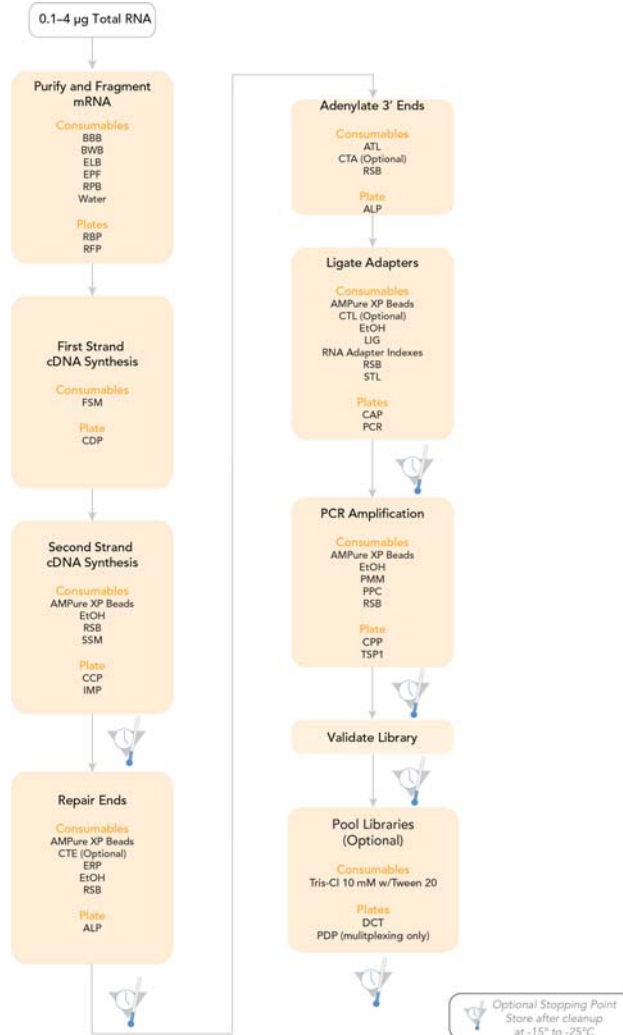
When processing 48 or fewer samples, Illumina recommends following the *Low-Throughput (LT) Protocol*.

The HT protocol requires shaking and heating equipment to mix reagents and for incubation (see *User-Supplied Consumables - Additional Items for HT Processing* and *User-Supplied Equipment - Additional Items for HT Processing*).

Sample Prep Workflow

The following illustrates the processes of the HT TruSeq RNA Sample Preparation protocol to prepare a template composed of 12 pooled indexed libraries.

Figure 12 TruSeq RNA Sample Preparation HT Workflow

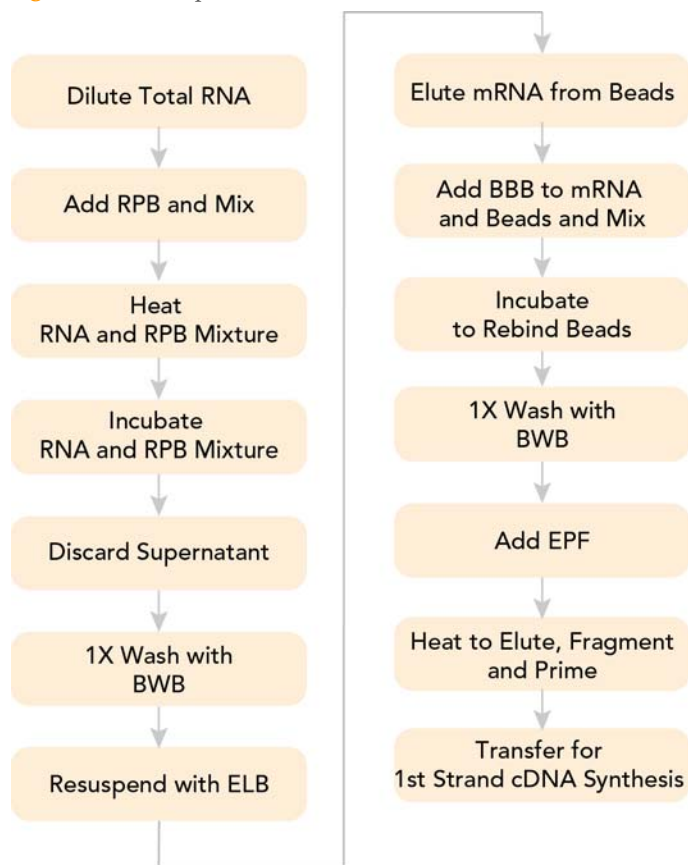


Purify and Fragment mRNA

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 13 TruSeq RNA Purification Workflow



It is important to follow this procedure exactly to ensure reproducibility.



NOTE

Allow the beads to fully pellet against the magnetic stand for at least 2 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* in this process.



NOTE

For inserts larger than 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)
- ▶ Bead Washing Buffer (BWB) (1 tube)
- ▶ Elution Buffer (ELB) (1 tube)
- ▶ Elute, Prime, Fragment Mix (EPF) (1 tube)
- ▶ RNA Purification Beads (RPB) (1 tube)
- ▶ RBP (RNA Bead Plate) barcode label
- ▶ RFP (RNA Fragmentation Plate) barcode label

User-Supplied Consumables

- ▶ 96-well MIDI plate
- ▶ 96-well TCY plate
- ▶ Microseal 'B' Adhesive Seal
- ▶ RNase/DNase-free Reagent Reservoirs

- ▶ RNase/DNase-free Strip Tubes and Caps
- ▶ Ultra Pure Water

Preparation

- ▶ For first time use, remove one tube each of Bead Binding Buffer, Bead Washing Buffer, Elution Buffer, and Elute, Prime, Fragment Mix from -15° to -25°C storage and thaw them at room temperature.
- ▶ 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**
- ▶ 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 TCY plate.



NOTE

- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.



NOTE

After use in this procedure, store the Bead Binding Buffer, Bead Washing Buffer, and Elution Buffer store 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 with the RBP barcode label.
- 2 Vortex the thawed RNA Purification Beads tube vigorously to completely resuspend the oligo-dT beads.

- 3 Add 50 μ l of RNA Purification Beads to each well of the RBP plate using a multichannel pipette to bind the poly-A RNA to the oligo dT magnetic beads. Change the tips after each column. 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 2 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 using a multichannel pipette. Take care not to disturb the beads. Change the tips after each column.
- 4 Remove the RBP plate from the magnetic stand.
- 5 Wash the beads by adding 200 μ l of Bead Washing Buffer in each well of the RBP plate using a multichannel pipette to remove unbound RNA. Change the tips after each column. 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 2 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 using a multichannel pipette. Take care not to disturb the beads. Change the tips after each column.
- 10 Remove the RBP plate from the magnetic stand.
- 11 Add 50 μ l of Elution Buffer in each well of the RBP plate using a multichannel pipette. Change the tips after each column. 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.
- 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 μ l of Bead Binding Buffer to each well of the RBP plate using a multichannel pipette to allow the RNA to re-bind to the beads. Change the tips after each column. 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 2 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 using a multichannel pipette. Take care not to disturb the beads. Change the tips after each column.
- 6 Remove the RBP plate from the magnetic stand.
- 7 Wash the beads by adding 200 μ l of Bead Washing Buffer in each well of the RBP plate using a multichannel pipette. Change the tips after each column. 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 2 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 using a multichannel pipette. Take care not to disturb the beads. Change the tips after each column.
- 12 Remove the RBP plate from the magnetic stand.
- 13 Add 19.5 μ l of Elute, Prime, Fragment Mix to each well of the RBP plate using a multichannel pipette. Change the tips after each column. 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 TCY plate labeled with the RFP barcode. Change the tips after each column.
- 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 Place the sealed RFP plate on the pre-programmed thermal cycler. Close the lid and select **Elution 2 - Frag - Prime** (94°C for 8 minutes, 4°C hold) to elute, fragment, and prime the RNA.
- 2 Remove the RFP plate from the thermal cycler when it reaches 4°C and centrifuge briefly.
- 3 Proceed immediately to *Synthesize First Strand cDNA*.

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 TCY 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 TCY 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. 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.

**NOTE**

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Make CDP

- 1 Place the RFP plate on the magnetic stand at room temperature for 2 minutes. Do not remove the plate from the magnetic stand.
- 2 Remove the adhesive seal from the RFP plate.
- 3 Transfer 17 μ l of the supernatant (fragmented and primed mRNA) from each well of the RFP plate to the corresponding well of the new TCY plate labeled with the CDP barcode. Some liquid may remain in each well.
- 4 Briefly centrifuge the thawed First Strand Master Mix to 600 xg for 5 seconds.
- 5 Add 50 μ l SuperScript II to the First Strand Master Mix tube (ratio: 1 μ l SuperScript II for each 7 μ l 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 μ l of First Strand Master Mix and SuperScript II mix to each well of the CDP plate using a multichannel pipette. Change the tips after each column. 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 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
- 2 When the thermal cycler reaches 4°C, remove the CDP plate from the thermal cycler and proceed immediately to *Synthesize Second Strand cDNA*.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand to generate double-stranded (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.
- ▶ 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.
- ▶ Review *AMPure XP Handling* on page 20.



NOTE

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

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 using a multichannel pipette. Change the tips after each column. 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 20 when working with AMPure XP Beads.

- 1 Vortex the AMPure XP beads until they are well dispersed, then add 90 μ l 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 tips after each column. 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 at least 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 μ l of the supernatant from each well of the CCP plate using a multichannel pipette. Some liquid may remain in each well. Take care not to disturb the beads. Change the tips after each column.

**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 μ l 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 using a multichannel pipette. Take care not to disturb the beads. Change the tips after each column.
- 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 \times g for 5 seconds.
- 12 Add 52.5 μ l Resuspension Buffer to each well of the CCP plate using a multichannel pipette. 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.

- 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 μ l of the supernatant (ds cDNA) from the CCP plate to the new MIDI plate labeled with the IMP barcode. Some liquid may remain in each well.



SAFE STOPPING POINT

If you do not plan to proceed to *Perform End Repair* immediately, the protocol can be safely stopped here. If you are stopping, seal the IMP plate with a Microseal 'B' adhesive seal and store it 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 (ERP) 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 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 of End Repair Mix and, if using End Repair Control, one tube of End Repair Control per 48 reactions from -15° to -25°C storage and thaw them at room temperature.



NOTE

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



NOTE

If you do not intend to consume the End Repair Control and End Repair Mix reagents in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- ▶ Remove the IMP plate from -15° to -25°C storage if it was stored at the conclusion of *Synthesize Second Strand cDNA* and let stand to thaw at room temperature.
- ▶ Review *AMPure XP Handling* on page 20.
- ▶ 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 ALP barcode label to a new 96-well MIDI plate.



NOTE

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Make IMP

- 1 If using the in-line control End Repair Control, briefly centrifuge the thawed End Repair Control tube to 600 xg for 5 seconds and 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.
- 2 Add 10 µl of diluted End Repair Control (or 10 µl of Resuspension Buffer if not using End Repair Control) to each well of the IMP plate that contains 50 µl of ds cDNA using a multichannel pipette. Change the tips after each column.
- 3 Add 40 µl of End Repair Mix to each well of the IMP plate containing the ds cDNA and End Repair Control using a multichannel pipette. 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, at 30°C for 30 minutes.
- 2 Remove the IMP plate from the microheating system.

Clean Up IMP



NOTE

Before performing clean up, review *AMPure XP Handling* on page 20 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 of 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 at least 5 minutes, until the liquid appears clear.
- 5 Remove the adhesive seal from the IMP plate.
- 6 Using a 200 µl multichannel pipette set to 127.5 µl, remove and discard 127.5 µl of the supernatant from each well of the IMP plate. Take care not to disturb the beads. Change the tips after each column.
- 7 Repeat step 6 once. Some liquid may remain in each well.



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 without disturbing the beads.

- 9 Incubate the IMP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tips after each column.
- 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 and then remove the plate from the magnetic stand.
- 12 Resuspend the dried pellet in 17.5 μ l 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.
- 13 Incubate the IMP plate at room temperature for 2 minutes.
- 14 Place the IMP plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
- 15 Remove the adhesive seal from the IMP plate.
- 16 Transfer 15 μ l 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. Some liquid may remain in each well.



SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3' Ends* immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store it 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) (1 tube)

User-Supplied Consumables

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

Preparation

- ▶ Remove one tube of A-Tailing Mix and, if using A-Tailing Control, one tube of A-Tailing Control per 48 reactions from -15° to -25°C storage and thaw them at room temperature.



NOTE

If you do not intend to consume the A-Tailing Mix and A-Tailing Control reagents in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- ▶ Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Perform End Repair* and let stand to thaw at room temperature.
- ▶ Briefly centrifuge the thawed ALP plate to 280 xg for 1 minute, then remove the adhesive seal from the plate.
- ▶ Pre-heat the microheating system to 37°C.



NOTE

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Add ATL

- 1 If using the in-line control A-Tailing Control, briefly centrifuge the thawed A-Tailing Control tube to 600 xg for 5 seconds and dilute the A-Tailing Control to 1/100 in Resuspension Buffer (1 μ l A-Tailing Control + 99 μ l Resuspension Buffer) before use. Discard the diluted A-Tailing Control after use.
- 2 Add 2.5 μ l of diluted A-Tailing Control (or 2.5 μ l of Resuspension Buffer if not using A-Tailing Control) to each well of the ALP plate using a multichannel pipette. 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.
 - d Remove the adhesive seal from the ALP plate.
- 3 Add 12.5 μ l of thawed A-Tailing Mix to each well of the ALP plate using a multichannel pipette. Change the tips after each column.
- 4 Seal the ALP plate with a Microseal 'B' adhesive seal.

Incubate 1 ALP

- 1 Incubate the ALP plate on the pre-heated microheating system, with the lid closed, at 37°C for 30 minutes.
- 2 Immediately remove the ALP plate from the microheating system, then proceed immediately to *Ligate Adapters*.

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

- ▶ DNA Ligase Mix (LIG) (1 tube per 48 reactions)
- ▶ (Optional) Ligase Control (CTL) (1 tube per 48 reactions)
- ▶ Resuspension Buffer (RSB) (1 tube)
- ▶ RNA Adapter Indexes 1–12 (AR001–AR012) (1 tube of each, depending on the RNA Adapter Indexes being used)
- ▶ Stop Ligase Mix (STL)
- ▶ CAP (Clean Up ALP Plate) barcode label
- ▶ PCR (Polymerase Chain Reaction) barcode label

User-Supplied Consumables

- ▶ 96-well MIDI plate
- ▶ 96-well TCY 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 appropriate RNA Adapter Index tubes (AR001–AR012, depending on the RNA Adapter Indexes being used), and one tube Stop Ligase Mix and, if using Ligase Control, one tube of Ligase Control, per 48 reactions from -15° to -25°C storage and thaw them at room temperature.



NOTE

If you do not intend to consume the Ligase Control in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- ▶ Review *AMPure XP Handling* on page 20.
- ▶ 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 TCY plate.



NOTE

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Add LIG

- 1 Briefly centrifuge the thawed RNA Adapter Index tubes (AR001–AR012 depending on the RNA Adapter Indexes being used), Ligase Control (if using Ligase Control), and Stop Ligase Mix tubes to 600 xg for 5 seconds.
- 2 Immediately before use, remove the DNA Ligase Mix tube from -15° to -25°C storage.



NOTE

If you do not intend to consume the DNA Ligase Mix in one use, dispense the reagent into single use aliquots and freeze in order to avoid repeated freeze thaw cycles.

- 3 Remove the adhesive seal from the ALP plate.
- 4 Add 2.5 µl of DNA Ligase Mix to each well of the ALP plate.
- 5 Return the DNA Ligase Mix tube back to -15° to -25°C storage immediately after use.
- 6 If using the in-line control Ligase Control, dilute the Ligase Control to 1/100 in Resuspension Buffer (1 µl Ligase Control + 99 µl Resuspension Buffer) before use. Discard the diluted Ligase Control after use.

- 7 Add 2.5 μ l of diluted Ligase Control (or 2.5 μ l of Resuspension Buffer if not using Ligase Control) to each well of the ALP plate.
- 8 Add 2.5 μ l of each thawed RNA Adapter Index (AR001–AR012 depending on the RNA Adapter Indexes being used) to each well of the ALP plate using a multichannel pipette. 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.

**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.

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 STL to each well of the ALP plate to inactivate the ligation mix using a multichannel pipette. 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 20 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 using a multichannel pipette. 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 at least 5 minutes, 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 using a multichannel pipette. Some liquid may remain in each well. Take care not to disturb the beads. Change the tips after each column.



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 μ l of freshly prepared 80% EtOH to each well without disturbing the beads.
- 8 Incubate the ALP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tips after each column.
- 9 Repeat steps 7 and 8 once for a total of two 80% EtOH washes.
- 10 Let the ALP plate stand at room temperature for 15 minutes to dry and then remove the plate from the magnetic stand.
- 11 Resuspend the dried pellet in each well with 52.5 μ l 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.
 - c Incubate the ALP plate at room temperature for 2 minutes.
- 12 Place the ALP plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
- 13 Remove the adhesive seal from the ALP plate.
- 14 Transfer 50 μ l 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. Some liquid may remain in each well. Change the tips after each column.
- 15 Vortex the AMPure XP Beads until they are well dispersed, then add 50 μ l of mixed AMPure XP Beads to each well of the CAP plate for a second clean up using a multichannel pipette. 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.
- 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 the adhesive seal from the CAP plate.
- 19 Remove and discard 95 μ l of the supernatant from each well of the CAP plate, using a multichannel pipette. Some liquid may remain in each well. Take care not to disturb the beads. Change the tips after each column.



NOTE

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

- 20 With the CAP plate remaining on the magnetic stand, add 200 μ l of freshly prepared 80% EtOH to each well without disturbing the beads.
- 21 Incubate the CAP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tips after each column.
- 22 Repeat steps 20 and 21 once for a total of two 80% EtOH washes.
- 23 Let the CAP plate stand at room temperature for 15 minutes to dry and then remove the plate from the magnetic stand.
- 24 Resuspend the dried pellet in each well with 22.5 μ l 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.
 - c Incubate the CAP plate at room temperature for 2 minutes.
- 25 Place the CAP plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
- 26 Remove the adhesive seal from the CAP plate.
- 27 Transfer 20 μ l of the clear supernatant from each well of the CAP plate to the corresponding well of the new TCY plate labeled with the PCR barcode. Some liquid may remain in each well. Change the tips after each column.



SAFE STOPPING POINT

If you do not plan to proceed to *Enrich DNA Fragments* immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store it 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)
- ▶ PCR Primer Cocktail (PPC) (1 tube)
- ▶ Resuspension Buffer (RSB) (1 tube)
- ▶ CPP (Clean Up PCR Plate) barcode label
- ▶ TSP1 (Target Sample Plate) barcode label

USER-SUPPLIED CONSUMABLES

- ▶ 96-well MIDI plate
- ▶ 96-well TCY 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 one tube each of PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature.
- ▶ Briefly centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- ▶ Review *AMPure XP Handling* on page 20.
- ▶ 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 *Ligate Adapters* 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 4°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 TCY plate.



NOTE

- Aliquot the appropriate volume of each reagent (with 10% excess volume) into strip tubes. Cap the tubes and keep them on ice until further use. The remaining content from the stock tube can be re-stored at -15° to -25°C.
- Take care to pipette accurately into the wells, as variations in volume will affect the sample preparation.
- Change tips after each column.
- Use RNase/DNase-free reagent reservoirs for beads and wash solutions.

Make PCR

- 1 Add 5 μ l of thawed PCR Primer Cocktail to each well of the PCR plate using a multichannel pipette. Change the tips after each column.
- 2 Add 25 μ l of thawed PCR Master Mix to each well of the PCR plate using a multichannel pipette. Change the tips after each column. Mix thoroughly as follows:
 - a Seal the PCR plate with a Microseal 'B' adhesive seal.
 - b Shake the PCR plate on a microplate shaker at 1,600 rpm for 20 seconds.
 - c Centrifuge the PCR plate to 280 xg for 1 minute.

Amp PCR

- 1 Amplify the PCR plate in the pre-programed thermal cycler, with the lid closed, using the **PCR** program:
 - 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 4°C

Clean Up PCR



NOTE

Before performing clean up, review *AMPure XP Handling* on page 20 when working with AMPure XP Beads.

- 1 Vortex the AMPure XP Beads until they are well dispersed, then add 50 μ l of the mixed AMPure XP Beads to each well of the new MIDI plate labeled with the CPP barcode using a multichannel pipette.
- 2 Centrifuge the PCR plate to 280 xg for 1 minute.

- 3 Transfer the entire contents from each well of the PCR plate to the corresponding well of the CPP plate containing 50 μ l of mixed AMPure XP Beads using a multichannel pipette. 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 at least 5 minutes, until the liquid appears clear.
- 6 Remove the adhesive seal from the CPP plate.
- 7 Remove and discard 95 μ l of the supernatant from each well of the CPP plate, using a multichannel pipette. Some liquid may remain in each well. Take care not to disturb the beads. Change the tips after each column.



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 μ l of freshly prepared 80% EtOH to each well without disturbing the beads.
- 9 Incubate the CPP plate at room temperature for at least 30 seconds, then remove and discard all of the supernatant from each well. Take care not to disturb the beads. Change the tips after each column.
- 10 Repeat steps 8 and 9 once for a total of two 80% EtOH washes.
- 11 Let the CPP plate stand at room temperature for 15 minutes to dry and then remove the plate from the magnetic stand.
- 12 Resuspend the dried pellet in each well with 32.5 μ l Resuspension Buffer using a multichannel pipette. 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.
 - c Incubate the CPP plate at room temperature for 2 minutes.
- 13 Place the CPP plate on the magnetic stand at room temperature for at least 5 minutes, until the liquid appears clear.
- 14 Remove the adhesive seal from the CPP plate.

- 15 Transfer 30 μl of the clear supernatant from each well of the CPP plate to the corresponding well of the new TCY plate labeled with the TSP1 barcode. Some liquid may remain in each well. Change the tips after each column.



SAFE STOPPING POINT

If you do not plan to proceed to *Validate Library* immediately, the protocol can be safely stopped here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store it 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 *qPCR Quantification Protocol Guide*.

Quality Control

- 1 Load 1 μ l 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 14 Example of TruSeq RNA Library Size Distribution

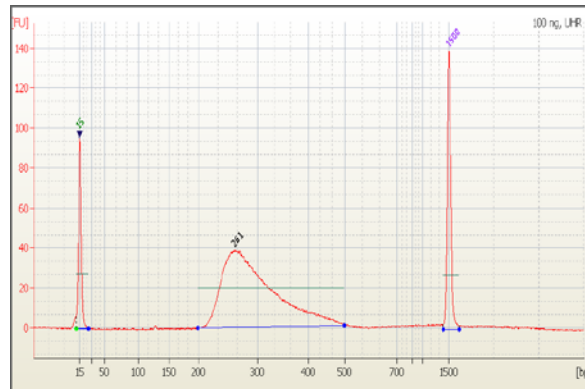
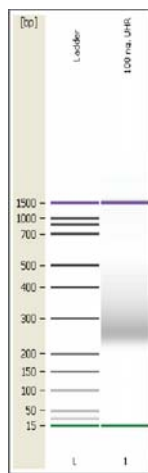


Figure 15 TruSeq RNA 260 bp PCR Product



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 (Diluted Cluster Template) plate and then pooled in equal volumes in the PDP (Pooled DCT 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 TCY 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 TCY plate (for multiplexing only).
- ▶ Remove the TSP1 plate from -15° to -25°C storage 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 1,800 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:
 - a 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 it at -15° to -25°C .
 - b For multiplexed paired-end libraries, proceed to *Make PDP*.

Make PDP

**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 TCY plate labeled with 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
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:
 - a Proceed to cluster generation. See the *Illumina Cluster Generation User Guide*.
 - b Seal the PDP plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C .

Alternate Fragmentation Protocols

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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; 1) modify the fragmentation time, or 2) shear the sample after the synthesis of the double-stranded 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 12.

Table 12 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* below.

Fragment Samples After ds cDNA Synthesis

To shear the sample after the synthesis of the double-stranded cDNA, during the *Purify and Fragment mRNA* procedure, 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 double-stranded 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 may 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 at 600 xg for 5 seconds.
- 4 Transfer contents to IMP plate and proceed to *Perform End Repair*.

Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 13 Illumina General Contact Information

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

Table 14 Illumina Customer Support Telephone Numbers

Region	Contact Number
North America toll-free	1.800.809.ILMN (1.800.809.4566)
United Kingdom toll-free	0800.917.0041
Germany toll-free	0800.180.8994
Netherlands toll-free	0800.0223859
France toll-free	0800.911850
Other European time zones	+44.1799.534000
Other regions and locations	1.858.202.ILMN (1.858.202.4566)

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. 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>.

