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TruSeq® Stranded mRNA Sample Preparation Guide

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Part # 15031047 Rev. D
September 2012

Revision History

Part #	Revision	Date	Description of Change
15031048	C	September 2012	• Added New England Biolabs, Inc. licensing to notices • Clarified that when starting with previously isolated mRNA, begin the protocol at the Incubate RFP procedures • Corrected PCR Primer Cocktail part number in LT Kit Contents • Corrected kit name with 96 Sample, cDNA Synthesis-PCR Box • Reformatted the consumables list at the start of each procedure to a table • After initial thaw, for each process that uses Resuspension Buffer, added a preparation step to remove it from 2° to 8°C storage

15031048	B	July 2012	• AddedTruSeq Stranded mRNA HT Sample Prep Kitcontent and functionality to the following sections:
			  • *Usage Guidelines*
			  • *Kit Contents*
			  • *Indexed Adapter Sequences*
			  • *Adapter Options*
			  • *Pooling Guidelines*
			  • *Ligate Adapters*procedures
			  • *Enrich DNA Fragments*procedures
			  • *Normalize and Pool Libraries s*procedures
			• Added reagent volume table to*Usage Guidelines*
			• Revised*Tracking Tools*documentation download information
			• Removed detailed Sample Sheet description from*Tracking Tools*
			• Added instructions for which assay to select when using the Illumina Experiment Manager
			• Corrected storage temperature for rRNA Binding Buffer and Elution Buffer as 2° to 8°C

Part #	Revision	Date	Description of Change
15031048	B (continued)	July 2012	• Added optional Agilent RNA 6000 Nano or Pico kits for alternative fragmentation to *Consumables and Equipment* list • Specified storage temperature for Resuspension Buffer at 2° to 8°C after initial thaw • Make RRP - Added steps to transfer supernatant from RIP to SIP plate and incubate
15031048	A	April 2012	Initial Release

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Overview

Chapter 1

Introduction

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

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 have the addition of a single 'A' base and subsequent ligation of the adapter. The products are then purified and enriched with PCR to create the final cDNA library.

The sample preparation protocol offers:

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

The protocol is compatible with no indexing or a lower indexing pooling level. The libraries generated do not require PCR amplification to enable cluster generation, although PCR is recommended in the standard protocol to robustly meet the yield requirements of most standard applications.

Audience and Purpose

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

- Chapter 3 Low Sample (LS) Protocol explains how to perform the TruSeq Stranded mRNA Sample Preparation using the Low Sample (LS) Protocol
- Chapter 4 High Sample (HS) Protocol explains how to perform the TruSeq Stranded mRNA Sample Preparation using the High Sample (HS) Protocol

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

Table 1 Protocol Features

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

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

Table 2 Kit and Sample Number Recommendations

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

Table 3 Kit and Protocol Recommendations

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

Getting Started

Chapter 2

Introduction

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

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

Acronyms

Table 4 TruSeq Stranded mRNA 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	Ligation Control
DCT	Diluted Cluster Template
ds cDNA	Double-Stranded Complimentary DNA
ELB	Elution Buffer
EUC	Experienced User Card
FPF	Fragment, Prime, Finish Mix
FSA	First Strand Synthesis Act D Mix

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

Best Practices

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

NOTE

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

Handling RNA

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

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

Handling Liquids

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

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

Handling Master Mix Reagents

When handling the master mix reagents:

- Minimize freeze-thaw cycles. If you do not intend to consume the reagents in one use, dispense the reagent into aliquots after the initial thaw and refreeze the aliquots in order to avoid excessive freeze-thaw cycles. However, if you aliquot, you might not have enough reagents for the full number of reactions over multiple uses.
- Add reagents in the order indicated and avoid making master-mixes containing the in-line controls.
- Take care while adding the A-Tailing Mix (ATL) and Ligation Mix (LIG) due to the viscosity of the reagents.
- First Strand Synthesis Mix Act D (FSA) contains Actinomycin D, a toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region. Please refer to the product material safety data sheet (MSDS) for detailed environmental, health, and safety information. MSDSs are available for this kit on the Illumina website at http://www.illumina.com/msds.

Handling Magnetic Beads

Follow appropriate handling methods when working AMPure XP Beads:

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

- Prior to use, allow the beads to come to room temperature.
- Do not reuse beads. Always add fresh beads when performing these procedures.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous.
- **When performing the LS protocol**:
  - After adding the beads to the reaction, mix the solution gently and thoroughly by pipetting up and down 10 times, making sure the liquid comes in contact with the beads and that the beads are resuspended homogeneously.
  - Pipetting with the tips at the bottom of the well and not pipetting the entire volume of the solution helps prevent the solution from foaming. Excessive foaming leads to sample loss, because the foam is not transferred out of the plate efficiently.
- **When performing the HS protocol**, after adding the beads to the reaction, seal the plate and shake the plate on a microplate shaker at 1,800 rpm for 2 minutes. Repeat, if necessary, until the color of the mixture appears homogeneous after mixing.
- Take care to minimize bead loss which can impact final yields.
- Change the tips for each sample.
- Let the mixed samples incubate for 15 minutes at room temperature for maximum recovery.
- When aspirating the cleared solution from the reaction plate and wash step, it is important to keep the plate on the magnetic stand and to not disturb the separated magnetic beads. Aspirate slowly to prevent the beads from sliding down the sides of the wells and into the pipette tips.
- To prevent the carryover of beads after elution, approximately 2.5 µl of supernatant are left when the eluates are removed from the bead pellet.
- Prepare fresh 80% ethanol. Ethanol tends to absorb water from the air, therefore, fresh 80% ethanol should be prepared for optimal results.
- Be sure to remove all of the ethanol from the bottom of the wells, as it can contain residual contaminants.

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

Avoiding Cross-Contamination

Practice the following to avoid cross-contamination:

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

Temperature Considerations

Temperature is an important consideration for making cDNA libraries:

- Keep libraries at temperatures ≤37°C, except where specifically noted.
- Place reagents on ice after thawing at room temperature.
- When processing more than 48 samples manually, Illumina recommends processing the plate on a bed of ice whenever possible, especially during the enzymatic steps (when using the A-Tailing Mix and Ligation Mix). A large number of samples processed at room temperature may result in uneven catalytic activity, which can lead to reduced quality of the end product.

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

Usage Guidelines

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

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

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

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

Table 5 TruSeq Stranded mRNA Sample Prep Reagent Volumes

Reagent	Description	Volume per Sample (µl)
AR0XX or RAP	RNA Adapter tube or RNA Adapter Plate	2.5
ATL	A-Tailing Mix	12.5
BBB	Bead Binding Buffer	50
BWB	Bead Washing Buffer	400
CTA	A-Tailing Control	2.5 of 1/100 dilution
CTE	End Repair Control	5 of 1/50 dilution
CTL	Ligation Control	2.5 of 1/100 dilution

Reagent	Description	Volume per Sample (µl)
ELB	Elution Buffer	50
FPF	Fragment, Prime, Finish Mix	19.5
FSA	First Strand Synthesis Act D Mix	8
LIG	Ligation Mix	2.5
PMM	PCR Master Mix	25
PPC	PCR Primer Cocktail	5
RPB	RNA Purification Beads	50
SMM	Second Strand Marking Master Mix	20
STL	Stop Ligation Buffer	5

Preparing More Than 24 Samples

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

Sample Distribution

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

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

NOTE

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

Reagents in Reservoirs

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

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

- Elution Buffer
- Resuspension Buffer
- RNA Purification Beads

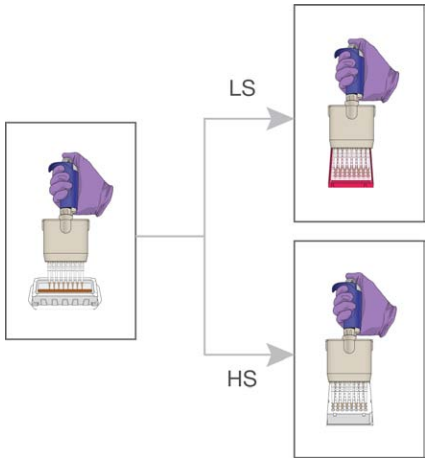
1 Determine the volume needed for each of the above reagents using the equation (# of samples x volume per sample) + 600 µl dead volume. Reference Table 5 or the protocol for the required reagent volume per sample.

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

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

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

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



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

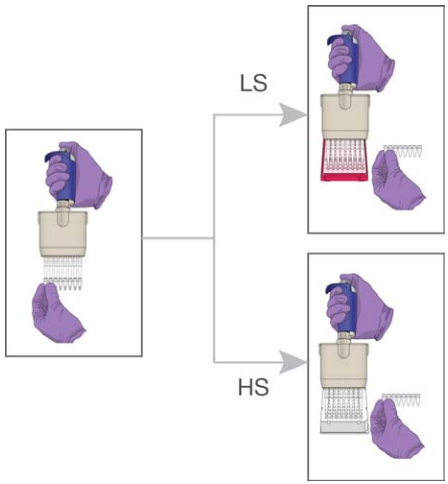
Reagents in Strip Tubes

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

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

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

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



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

Index Adapters

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

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

When using a RAP, see *Handling Adapter Plate* on page 44.

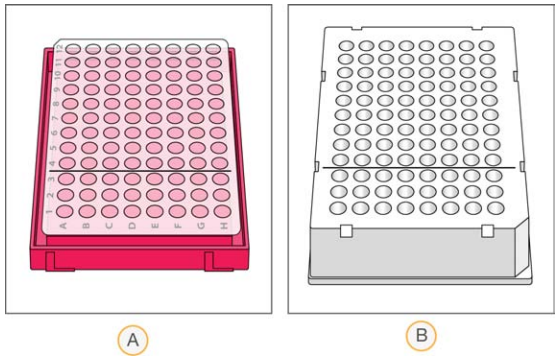
Preparing 12–24 Samples

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

Sample Distribution

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

Figure 3 Draw Line on Plate



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

Reagents in Reservoirs

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

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

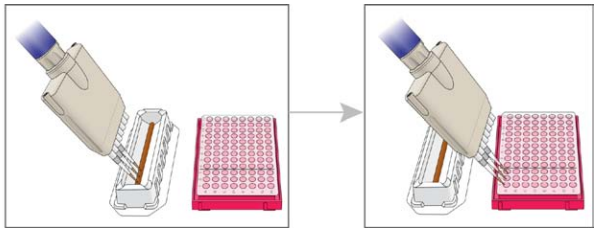
1 Determine the volume needed using the equation (# of samples x volume per sample) + 600 µl dead volume. Reference Table 5 or the protocol for the required reagent volume per sample.

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

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

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

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



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

Reagents in Deep Well Plates

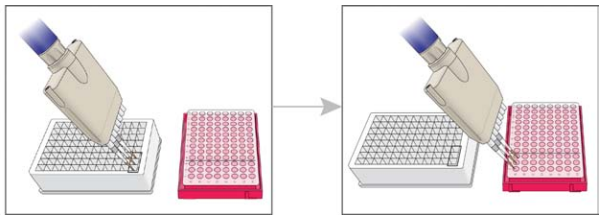
When each of the following reagents are required in the protocol, distribute each into three wells, in the same row of a deep well plate, adding 75 µl dead volume.

- RNA Purification Beads
- Elution Buffer
- Bead Binding Buffer

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

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

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



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

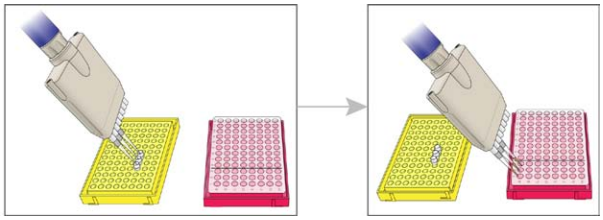
Reagents in Strip Tubes

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

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

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

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



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

Index Adapter Tubes

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

Preparing Less Than 12 Samples

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

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

Equipment

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

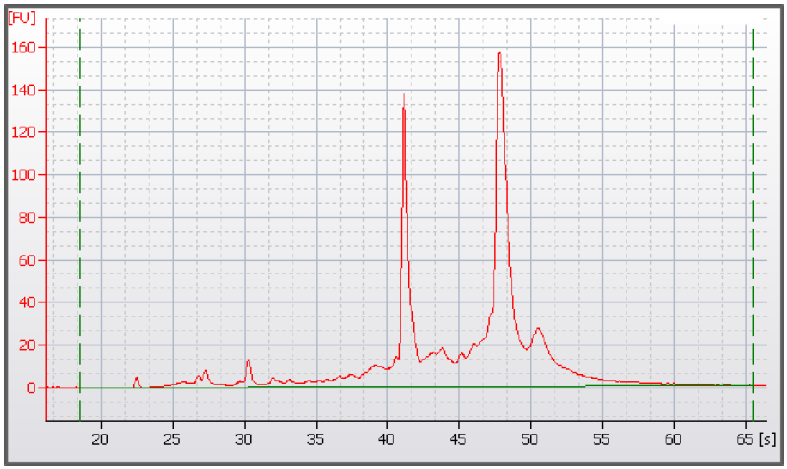
RNA Input Recommendations

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

Total RNA Input

- This protocol is optimized for 0.1–4 µg of total RNA.
  - Lower amounts might 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 might require further optimization with regard to the initial input amount.
- The protocol recommends diluting the in-line controls for tracking the steps involved in converting dsDNA into libraries.
  - The dilution is optimized for 0.1–4 µg of high quality input RNA.
  - When using less RNA or RNA with very low mRNA content, these controls might need further dilution.
  - If no controls are added, use Resuspension Buffer in place of the controls in the protocol.
- It is very important to know the quality of the RNA starting material. The fragmentation conditions were optimized for high quality RNA.
  - Illumina does not recommend the use of low quality or degraded RNA with this protocol. Use of degraded RNA can result in low yield, over-representation of the 3' ends of the RNA molecules, or failure of the protocol.
  - Illumina recommends that you check total RNA integrity following isolation using an Agilent Technologies 2100 Bioanalyzer for samples 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 7 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 *Incubate RFP* on page 66 for LS processing or *Incubate RFP* on page 103 for HS processing.

Positive Control

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

In-Line Control DNA

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

NOTE

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

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

Table 6 In-Line Control Functions

Reagent	Function	Control	Structure of Control DNA Ends
Second Strand Marking Master Mix	End repair: Generate blunt ended fragments by 3'–>5' exonuclease and polymerase activities	End Repair Control 1*	5' overhang at one end, 3' overhang at other end
Second Strand Marking Master Mix	End repair: Add 5'-phosphate groups needed for downstream ligation	End Repair Control 2*	Blunt with 5'-OH group

Reagent	Function	Control	Structure of Control DNA Ends
A-Tailing Mix	A-tailing: Make fragments compatible with adapters and prevent self-ligation by adding a 3'-A overhang	A-Tailing Control	Blunt with 5'-phosphate group
Ligation Mix	Ligation: Join adapters to inserts	Ligation Control	Single-base 3' 'A' base overhang

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

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

Tracking Tools

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

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

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

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

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

Kit Contents

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

Table 7 TruSeq Stranded mRNA Sample Preparation Kits

Kit Name	Catalog #	Number of Samples Supported	Number of Indices
TruSeq Stranded mRNA LT Sample Prep Kit - Set A	RS-122-2101	48	12
TruSeq Stranded mRNA LT Sample Prep Kit - Set B	RS-122-2102	48	12
TruSeq Stranded mRNA HT Sample Prep Kit	RS-122-2103	96	96

TruSeq Stranded mRNA LT Sample Prep Kit

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

48 Samples, 12 Index Set A and B

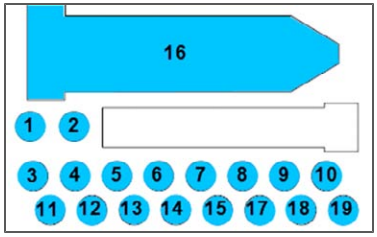
You will receive either box A or B in the kit, depending on the set ordered.

Store at -15° to -25°C

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

Set A

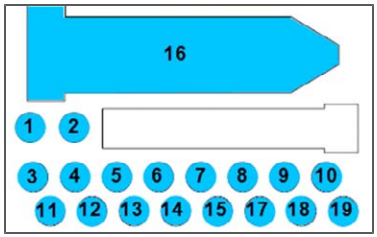
Figure 8 TruSeq Stranded mRNA LT Sample Prep Kit 48 Samples, 12 Index Set A, part # 15032612



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

Set B

Figure 9 TruSeq Stranded mRNA LT Sample Prep Kit 48 Samples, 12 Index Set B, part # 15032613



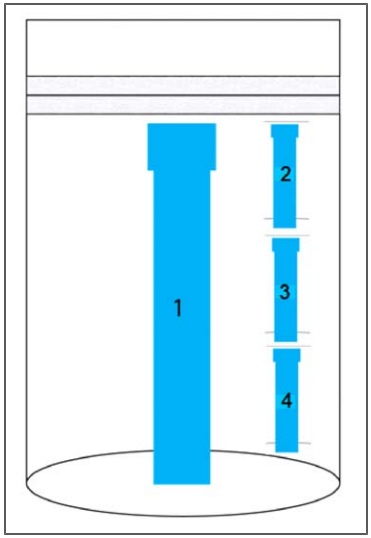
Slot	Reagent	Part #	Description
1	ATL	15012495	A-Tailing Mix
2	STL	15012546	Stop Ligation Buffer
3	AR020	15024662	RNA Adapter Index 20
4	AR021	15024663	RNA Adapter Index 21
5	AR022	15024664	RNA Adapter Index 22
6	AR023	15024665	RNA Adapter Index 23
7	AR025	15024667	RNA Adapter Index 25
8	AR027	15024668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

48 Samples, Box 1 of 2

Store as specified

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

Figure 10 TruSeq Stranded mRNA LT Sample Prep Kit 48 Samples, (Box 1 of 2), part # 15027078



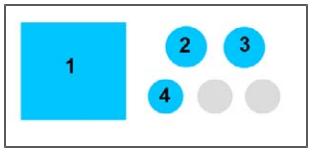
Slot	Reagent	Part #	Description	Storage Temperature
1	RPB	15032377	RNA Purification Beads	2° to 8°C
2	DTE	15026766	CTE Dilution Tube	Room Temperature
3	DTA	15026805	CTA Dilution Tube	Room Temperature
4	DTL	15026807	CTL Dilution Tube	Room Temperature

48 Samples, Box 2 of 2

Store as specified

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

Figure 11 TruSeq Stranded mRNA LT Sample Prep Kit 48 Samples, (Box 2 of 2), part # 15032614



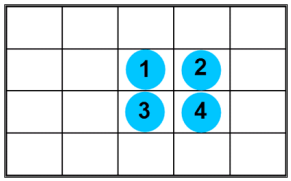
Slot	Reagent	Part #	Description	Storage Temperature
1	BWB	15012925	Bead Washing Buffer	2° to 8°C
2	BBB	15026779	Bead Binding Buffer	2° to 8°C
3	ELB	15026780	Elution Buffer	2° to 8°C
4	FPF	15032067	Fragment, Prime, Finish Mix	-15° to -25°C

48 Samples, cDNA Synthesis PCR Box

Store at -15° to -25°C

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

Figure 12 TruSeq Stranded mRNA LT Sample Prep Kit, 48 Samples, cDNA Synthesis PCR Box, part # 15032611



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

TruSeq Stranded mRNA HT Sample Prep Kit

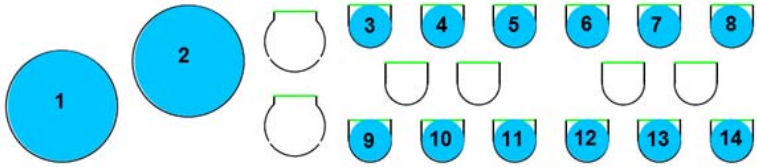
The TruSeq Stranded mRNA HT Sample Prep Kit contains five boxes: a core reagent box, a cDNA Synthesis- PCR box, an Adapter Plate box, and a Box 1 and Box 2.

96 Samples, Core Box

Store at -15° to -25°C

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

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



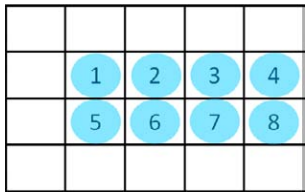
Slot	Reagent	Part #	Description
1–2	RSB	15026770	Resuspension Buffer
3–4	ATL	15012495	A-Tailing Mix
5–6	LIG	15026773	Ligation Mix
7–8	CTE	15026774	End Repair Control
9–10	CTA	15026775	A-Tailing Control
11–12	CTL	15026776	Ligation Control
13–14	STL	15012546	Stop Ligation Buffer

96 Samples, cDNA Synthesis-PCR Box

Store at -15° to -25°C

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

Figure 14 TruSeq Stranded mRNA HT Sample Prep Kit, 96 Samples, cDNA Synthesis-PCR Box, part # 15032621



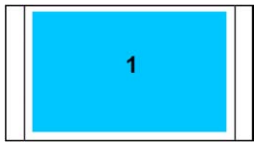
Slot	Reagent	Part #	Description
1–2	PMM	15026785	PCR Master Mix
3–4	PPC	15031748	PCR Primer Cocktail
5–6	FSA	15031094	First Strand Synthesis Act D Mix
7–8	SMM	15031098	Second Strand Marking Master Mix

96 Samples- Adapter Plate Box

Store at -15° to -25°C

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

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



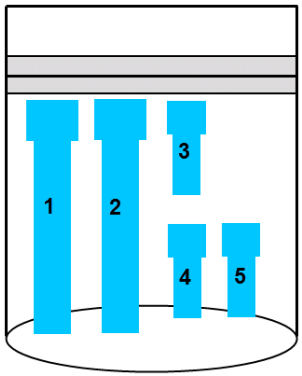
Slot	Reagent	Part #	Description
1	RAP	15016427	RNA Adapter Plate, 96plex

96 Samples, Box 1 of 2

Store as specified

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

Figure 16 TruSeq Stranded mRNA HT Sample Prep Kit, 96 Samples, (Box 1 of 2), part # 15032624



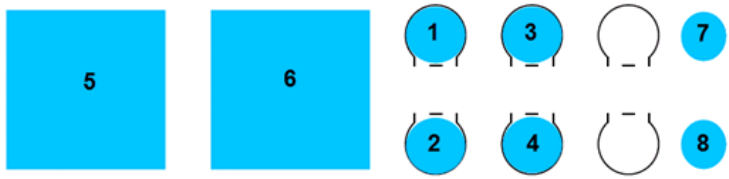
Slot	Reagent	Part #	Description	Storage Temperature
1–2	RPB	15026778	RNA Purification Beads	2° to 8°C
3	DTL	15026807	CTL Dilution Tube	Room Temperature
4	DTE	15026766	CTE Dilution Tube	Room Temperature
5	DTA	15026805	CTA Dilution Tube	Room Temperature

96 Samples, Box 2 of 2

Store as specified

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

Figure 17 TruSeq Stranded mRNA HT Sample Prep Kit, 96 Samples, (Box 2 of 2), part # 15032623



Slot	Reagent	Part #	Description	Storage Temperature
1–2	BBB	15026779	Bead Binding Buffer	2° to 8°C
3–4	ELB	15026780	Elution Buffer	2° to 8°C
5–6	BWB	15012925	Bead Washing Buffer	2° to 8°C
7–8	FPF	15032067	Fragment, Prime, Finish Mix	-15° to -25°C

Consumables and Equipment

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

Table 8 User-Supplied Consumables

Consumable	Supplier
1.5 ml RNase/DNase-free non-sticky tubes	Life Technologies, part # AM12450
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
96-well storage plates, round well, 0.8 ml (“MIDI” plate)	Fisher Scientific, part # AB-0859
96-well 2 ml deep well plates (Optional - for alliquotting reagents)	Thomson Instrument Company, part # 951652

Consumable	Supplier
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
Certified low-range ultra agarose (Optional - to determine input RNA integrity)	Bio-Rad, part # 161-3107
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma Aldrich, part # E7023
MicroTube (6x16mm), AFA fiber with crimp-cap (Optional - for alternative fragmentation only)	Covaris, part # 520052
Microseal 'B' adhesive seals	BioRad, part # MSB-1001
MinElute Gel Extraction Kit (Optional - if starting with previously isolated mRNA)	QIAGEN, part# 28604
Nuclease-free ultra pure water	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
RNase/DNase-free 8-well PCR strip tubes and caps	General lab supplier
RNase/DNase zapper (to decontaminate surfaces)	General lab supplier
SuperScript II Reverse Transcriptase	Invitrogen, part # 18064-014
Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20	General lab supplier
Tween 20	Sigma, part # P7949

Table 9 User-Supplied Consumables - Additional Items for LS Processing

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

Table 10 User-Supplied Consumables - Additional Items for HS Processing

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

Table 11 User-Supplied Equipment

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

Table 12 User-Supplied Equipment - Additional Items for HS Processing

Consumable	Supplier
High Speed Micro Plate Shaker	VWR, catalog # 13500-890 (110V/120V) catalog # 14216-214 (230V)
MIDI plate insert for heating system Note: Two inserts are recommended to support successive heating procedures.	Illumina, catalog # BD-60-601

Consumable	Supplier
Stroboscope	General lab supplier
Tru Temp Microheating System Note: Two systems are recommended to support successive heating procedures.	Illumina, catalog # SC-60-503 (115V) catalog # SC-60-504 (220V)

Indexed Adapter Sequences

This section details the indexed adapter sequences.

TruSeq Stranded mRNA LT Sample Prep Kit Indexed Adapter Sequences

The TruSeq Stranded mRNA LT Sample Prep Kit contains the following the indexed adapter sequences. The set (A or B) containing the adapter is also specified.

NOTE

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

Table 13 TruSeq Stranded mRNA LT Sample Prep Kit Indexed Adapter Sequences

Adapter	Sequence	Set		Adapter	Sequence	Set
AR001	ATCACG(A)	B		AR013	AGTCAA(C)	A
AR002	CGATGT(A)	A		AR014	AGTTCC(G)	A
AR003	TTAGGC(A)	B		AR015	ATGTCA(G)	A
AR004	TGACCA(A)	A		AR016	CCGTCC(C)	A
AR005	ACAGTG(A)	A		AR018	GTCCGC(A)	A
AR006	GCCAAT(A)	A		AR019	GTGAAA(C)	A
AR007	CAGATC(A)	A		AR020	GTGGCC(T)	B
AR008	ACTTGA(A)	B		AR021	GTTTCG(G)	B

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

TruSeq Stranded mRNA HT Sample Prep Kit Indexed Adapter Sequences

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

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

Table 14 TruSeq Stranded mRNA HT Sample Prep Kit Indexed Adapter Sequences

Indexed Adapter 1	Sequence	Indexed Adapter 2	Sequence
D701	ATTACTCG	D501	TATAGCCT
D702	TCCGGAGA	D502	ATAGAGGC
D703	CGCTCATT	D503	CCTATCCT
D704	GAGATTCC	D504	GGCTCTGA
D705	ATTCAGAA	D505	AGGCGAAG
D706	GAATTCGT	D506	TAATCTTA
D707	CTGAAGCT	D507	CAGGACGT

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

Adapter Options

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

Adapter Tubes

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

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

For more information on pooling guidelines when using adapter index tubes, see *Adapter Tube Pooling Guidelines* on page 47.

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

Adapter Plate

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

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

For more information on pooling guidelines when using the RAP, see *Adapter Plate Pooling Guidelines* on page 48.

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

Table 15 Dual-Indexed Sequencing Platform Compatibility

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

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

Pooling Preparation with Adapter Plate

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

Figure 18 RAP Dual-Indexed Layout

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

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

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

2 Ensure that the pool contains the required index combinations, as described in *Adapter Plate Pooling Guidelines* on page 48. Select the RNA index adapters based on the same guidelines.

3 Use the Illumina Experiment Manager to create a sample sheet which will be used during the sequencing run. This step also identifies any incorrect index combinations, allowing re-design before library preparation starts. For more information, see *Tracking Tools* on page 25.

4 Use the Lab Tracking Form or sample plate generator from the Illumina Experiment Manager to specify the layout of all sample plates in 96-well plate format for compatibility with the 96-well RAP. Arrange samples that will be pooled together in the same orientation as the indices in the RAP. For more information, see *Tracking Tools* on page 25.

Handling Adapter Plate

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

- The RAP is single-use for each well.

- Illumina recommends that the RAP does not undergo more than 4 freeze-thaw cycles.
- To maximize the use of the RAP, process more than 24 samples at a time. These samples can then be pooled in any supported configuration.

Prepare Adapter Plate

Prepare the RAP for use as follows:

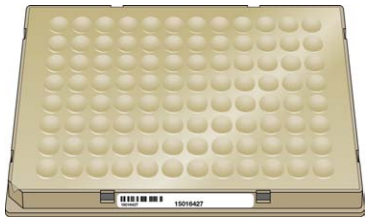
1. Thaw the plate for 10 minutes at room temperature on the benchtop. Visually inspect the wells to ensure that they all are completely thawed.
2. Remove the adapter plate tape seal.
3. Centrifuge the plate at 280 xg for 1 minute to collect all of the adapter to the bottom of the well.
4. Remove the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

Pierce Adapter Plate Seal

Pierce the RAP foil seal as follows:

1. Place the RAP on the benchtop so that the part number barcode on the long side of the plate is facing you and the clipped corner is located on the lower left.

   Figure 19 Correct RAP Orientation

2. Do one of the following:

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

Pipette Adapter Plate

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

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

Adapter Plate Storage

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

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

NOTE

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

Pooling Guidelines

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

Adapter Tube Pooling Guidelines

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

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

Table 16 Single-Indexed Pooling Strategies for 2–4 Samples

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

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

Adapter Plate Pooling Guidelines

When using the the RAP from the TruSeq Stranded mRNA HT Sample Prep Kit, follow these pooling guidelines. In addition, please review *Handling Adapter Plate* on page 44 and *Pooling Preparation with Adapter Plate* on page 43.

Single-Indexed Sequencing

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

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

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

Figure 20 Single-Indexed–2-plex

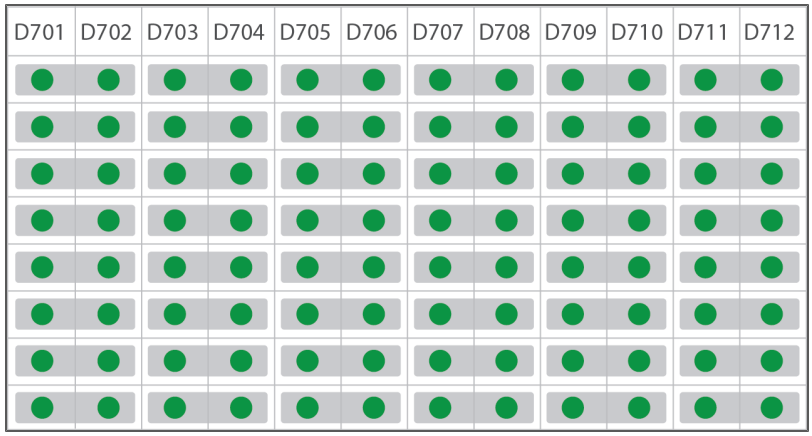


Figure 21 Single-Indexed–3-plex

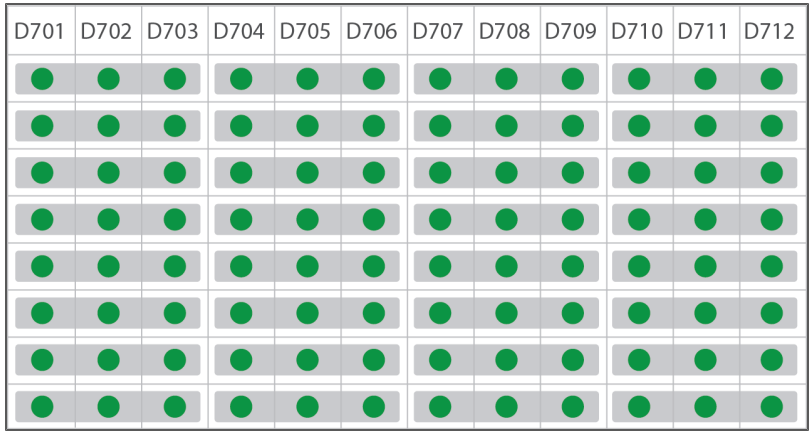


Figure 22 Single-Indexed–4-plex

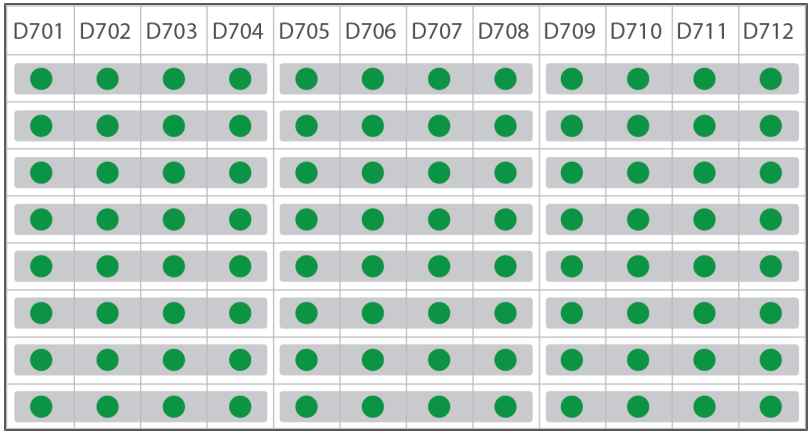


Figure 23 Single-Indexed–5-plex

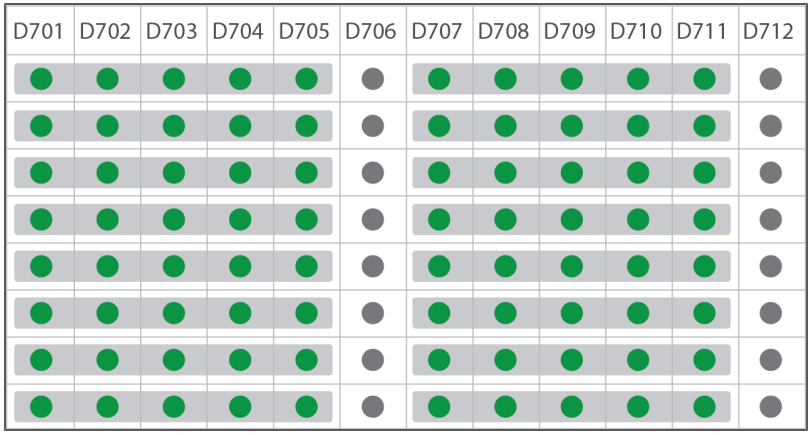


Figure 24 Single-Indexed–6-plex

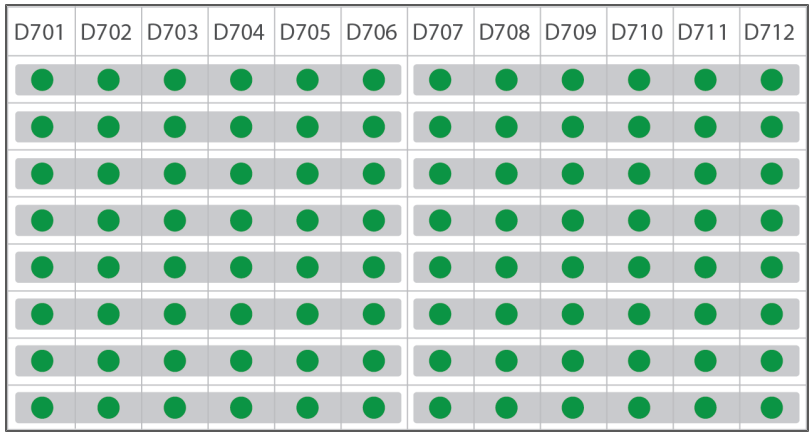
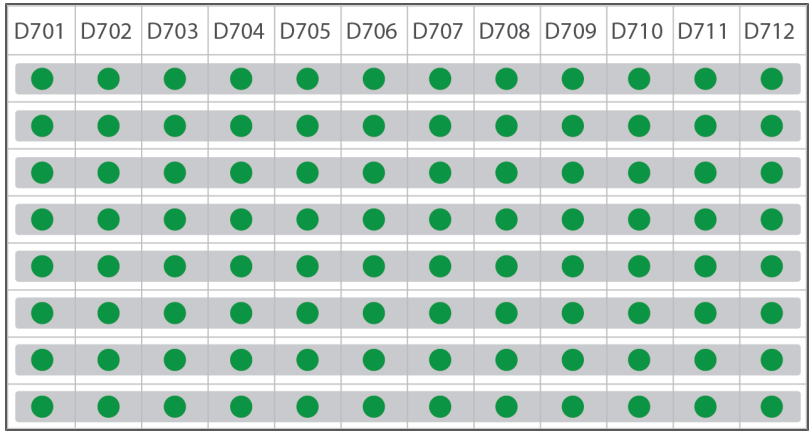


Figure 25 Single-Indexed–12-plex



Dual-Indexed Sequencing

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

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

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

Figure 26 Dual-Indexed–2-plex

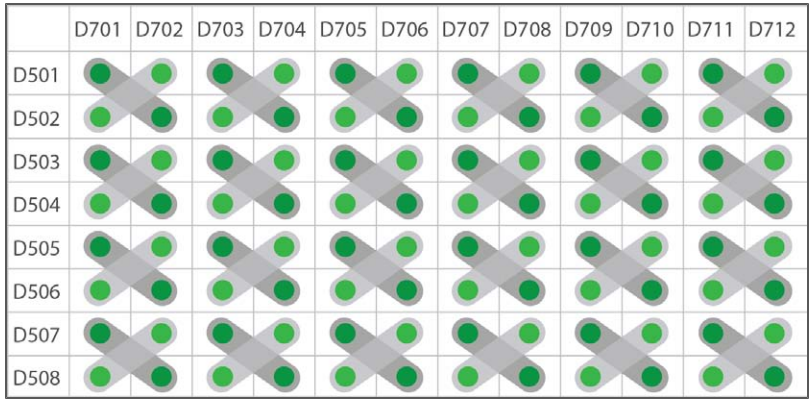


Figure 27 Dual-Indexed–3-plex

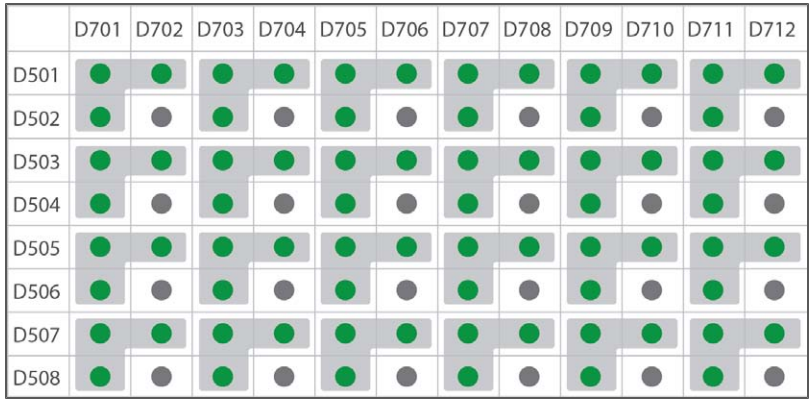


Figure 28 Dual-Indexed–4-plex

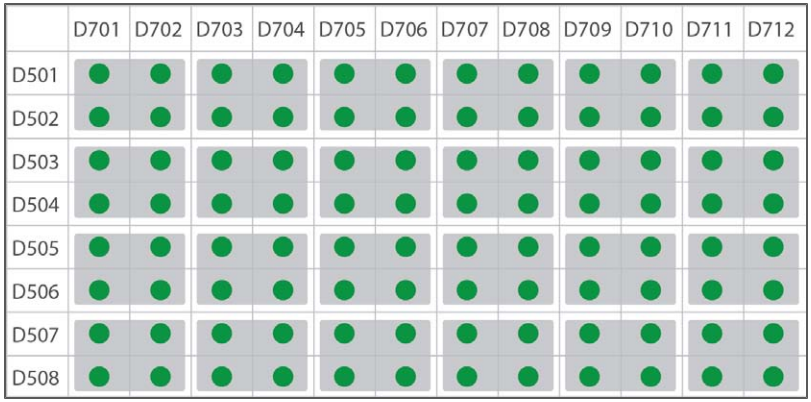


Figure 29 Dual-Indexed–5-plex

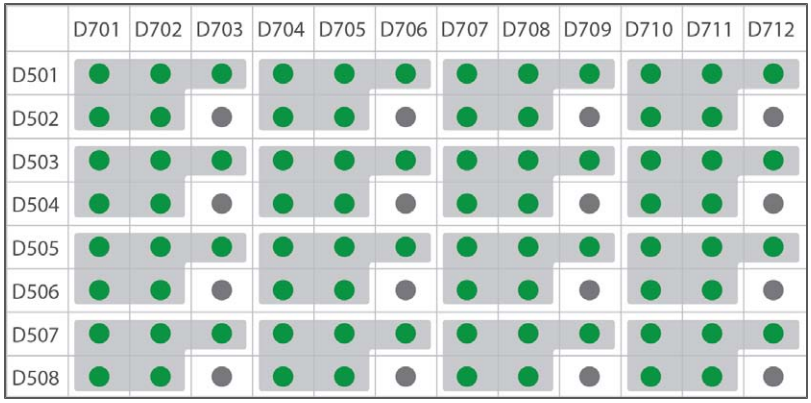


Figure 30 Dual-Indexed–6-plex

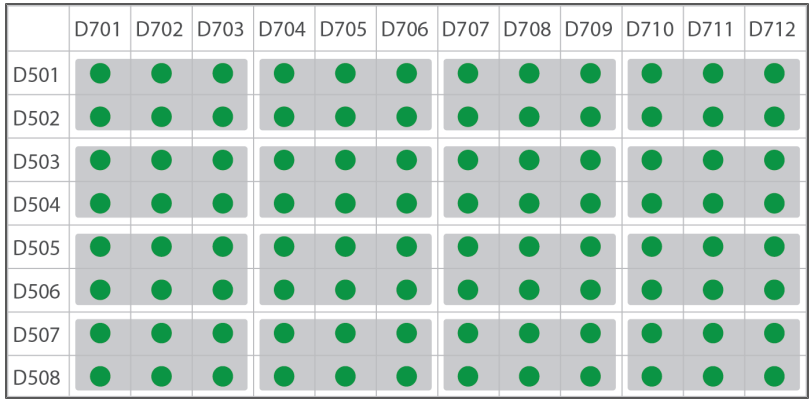


Figure 31 Dual-Indexed–7-plex

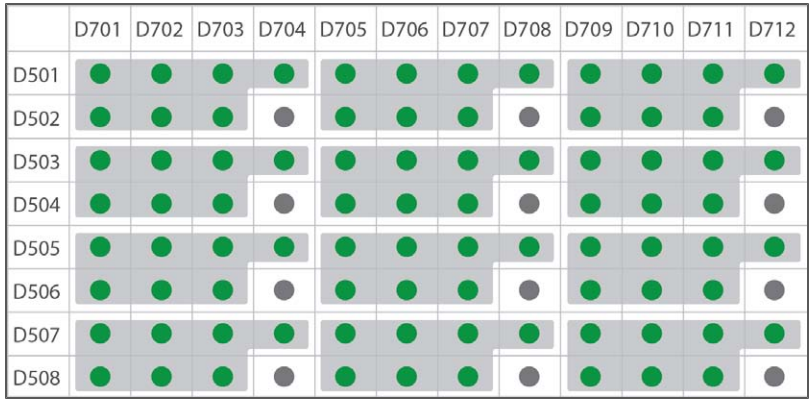


Figure 32 Dual-Indexed–8-plex, Option 1

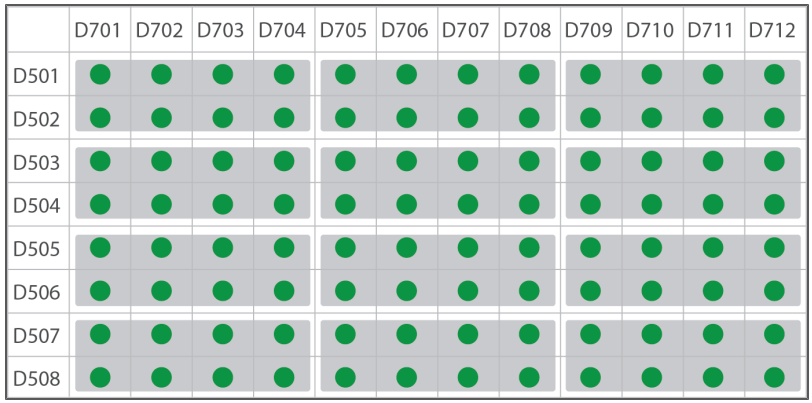


Figure 33 Dual-Indexed–8-plex, Option 2

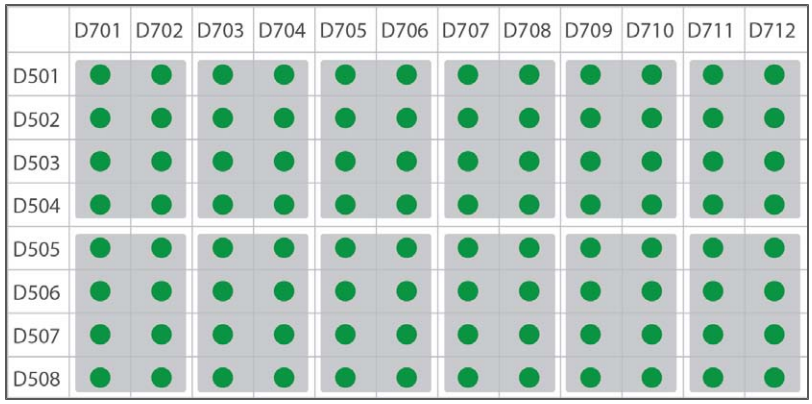


Figure 34 Dual-Indexed–12-plex

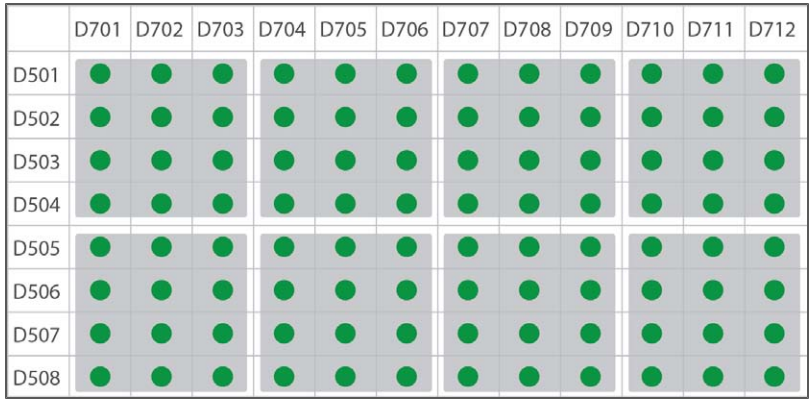
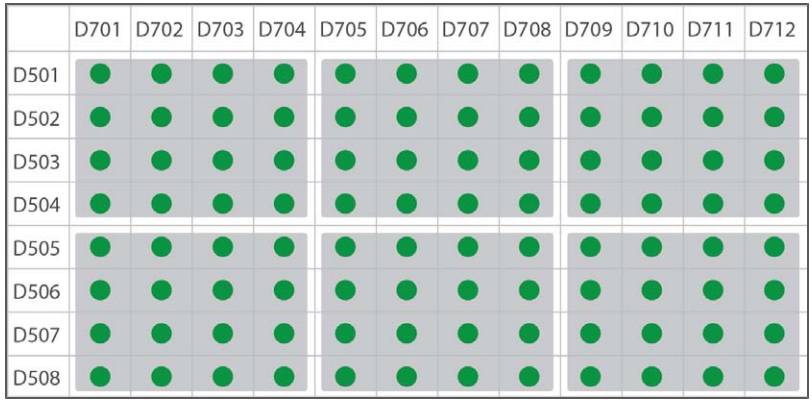
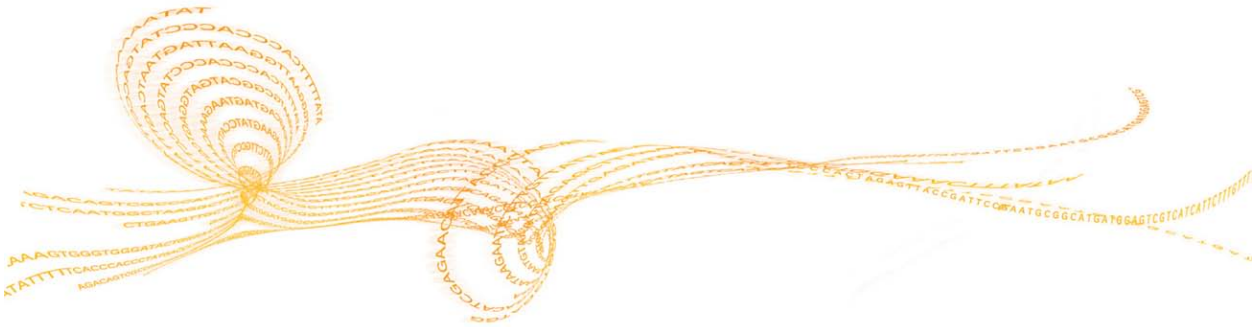


Figure 35 Dual-Indexed–16-plex



Low Sample (LS) Protocol

Chapter 3



Introduction

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

Table 17 Kit and Sample Number Recommendations

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

Table 18 Kit and Protocol Recommendations

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

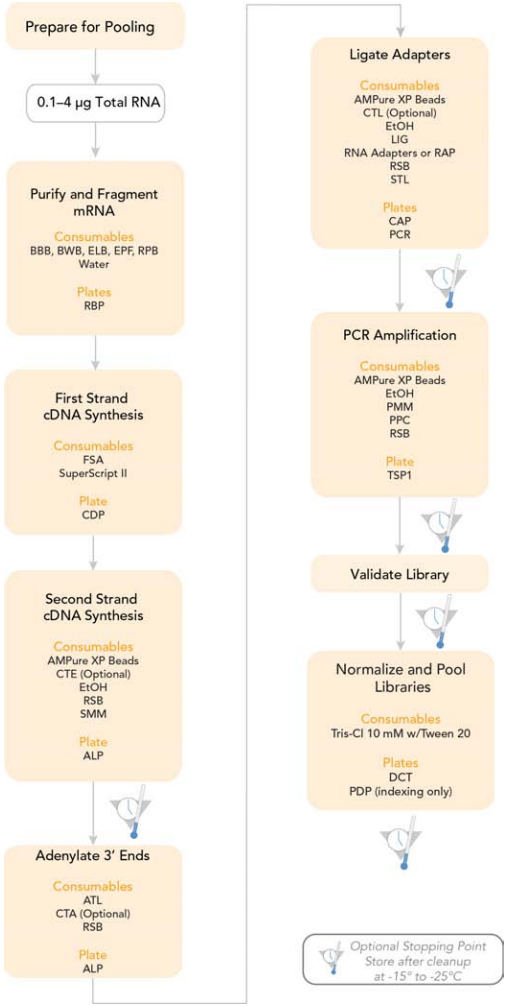
- Review *Best Practices* on page 9 before proceeding.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample preparation. For more information, see *Tracking Tools* on page 25.

- If you are pooling using adapter index tubes, record information about your samples before beginning library preparation for later use in data analysis. For more information, see *Tracking Tools* on page 25. Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
- If you are pooling with the RAP, please review the planning steps in *Pooling Preparation with Adapter Plate* on page 43 before beginning library preparation.

Sample Prep Workflow

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

Figure 36 TruSeq Stranded mRNA Sample Preparation LS Workflow

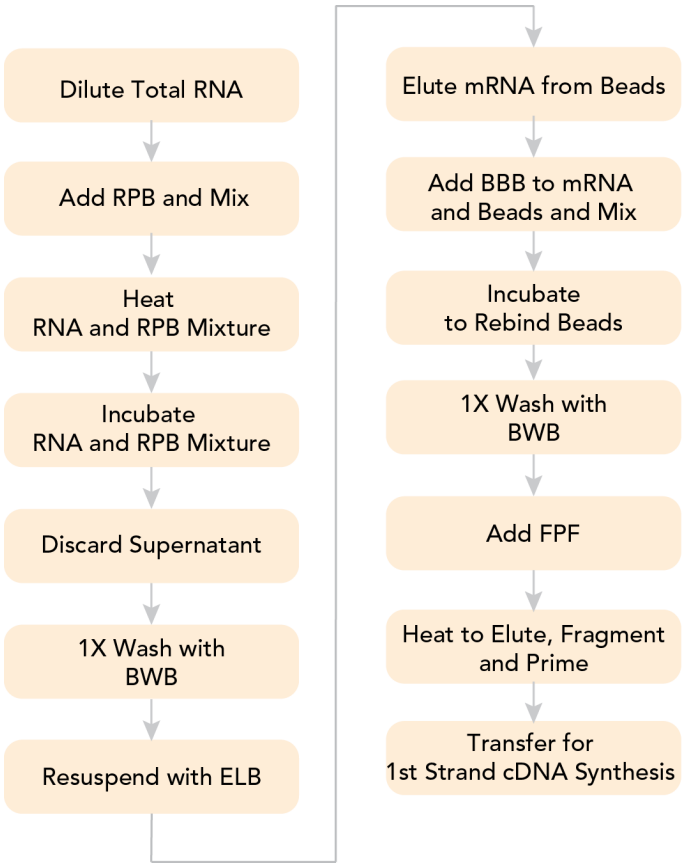


Purify and Fragment mRNA

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 37 TruSeq Stranded mRNA Sample Prep Purification Workflow



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

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

NOTE
Illumina recommends that you use 0.1–4 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, the mRNA must be concentrated into 5 µl or less before addition to Fragment, Prime, Finish Mix. This can be done by ethanol precipitation or by concentration on a Qiagen MinElute column.

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

In either case, heat the mRNA in Elute, Prime, Fragment Mix to fragment at *Incubate RFP* on page 66 in this process.

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

Consumables

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

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	-15° to -25°C	Illumina
RNA Purification Beads (RPB)	1 tube per 48 reactions	2° to 8°C	Illumina
RBP (RNA Bead Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well 0.3 ml PCR plate	1	15° to 30°C	User
Microseal 'B' Adhesive Seals	3	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	6	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	6	15° to 30°C	User

Preparation

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

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

- Remove the RNA Purification Beads tube from 2° to 8°C storage and let stand to bring to room temperature.
- Pre-program the thermal cycler with the following programs:
  - Choose the pre-heat lid option and set to 100°C
  - 65°C for 5 minutes, 4°C hold – save as **mRNA Denaturation**
  - 80°C for 2 minutes, 25°C hold – save as **mRNA Elution 1**

- 94°C for 8 minutes, 4°C hold – save as **Elution 2 - Frag - Prime**

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

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

Make RBP

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

Incubate 1 RBP

1. Place the sealed RBP plate on the pre-programmed thermal cycler. Close the lid and select **mRNA Denaturation** 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. Remove the adhesive seal from the RBP plate.
2. Place the RBP plate on the magnetic stand at room temperature for 5 minutes to separate the poly-A RNA bound beads from the solution.
3. Remove and discard all of the supernatant from each well of the RBP plate.

4 Remove the RBP plate from the magnetic stand.

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

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

7 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. The supernatant contains the majority of the ribosomal and other non-messenger RNA.

9 Remove the RBP plate from the magnetic stand.

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

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** to elute the mRNA from the beads. This releases both the mRNA and any contaminant rRNA that has bound the beads non-specifically.

2 Remove the RBP plate from the 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 Centrifuge the thawed Bead Binding Buffer to 600 xg for 5 seconds.

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

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

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

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

6 Remove the RBP plate from the magnetic stand.

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

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

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

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

11 Remove the RBP plate from the magnetic stand.

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

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

14 Store the Fragment, Prime, Finish 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** to elute, fragment, and prime the RNA.

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

3 Proceed immediately to *Synthesize First Strand cDNA* on page 67.

Synthesize First Strand cDNA

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

Consumables

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

WARNING

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

Preparation

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

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

Make CDP

1. Place the RBP plate on the magnetic stand at room temperature for 5 minutes. Do not remove the plate from the magnetic stand.
2. Remove the adhesive seal from the RBP plate.
3. Transfer 17 µl of the supernatant (fragmented and primed mRNA) from each well of the RBP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CDP barcode.
4. Centrifuge the thawed First Strand Synthesis Act D Mix tube to 600 xg for 5 seconds.
5. Add 50 µl SuperScript II to the First Strand Synthesis Act D Mix tube. If you are not using the entire contents of the First Strand Synthesis Act D Mix tube, add SuperScript II at a ratio of 1 µl SuperScript II for each 9 µl First Strand Synthesis Act D Mix. Mix gently, but thoroughly, and centrifuge briefly.

Label the First Strand Synthesis Act D Mix tube to indicate that the SuperScript II has been added.

6 Add 8 µl of First Strand Synthesis Act D Mix and SuperScript II mix to each well of the CDP plate. Gently pipette the entire volume up and down 6 times to mix thoroughly.

7 Seal the CDP plate with a Microseal 'B' Adhesive seal and centrifuge briefly.

8 Return the First Strand Synthesis Act D Mix tube to -15° to -25°C storage immediately after use.

Incubate 1 CDP

1 Place the sealed CDP plate on the pre-programmed thermal cycler. Close the lid and select the **Synthesize 1st Strand** program.

2 When the thermal cycler reaches 4°C, remove the CDP plate from the thermal cycler and proceed immediately to *Synthesize Second Strand cDNA* on page 70.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand, incorporating dUTP in place of dTTP to generate ds cDNA. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix. At the end of this process, you will have blunt-ended cDNA.

Consumables

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

Preparation

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

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

  - Second Strand Marking Master Mix
- Remove the Resuspension Buffer from 2° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 10.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the thermal cycler to 16°C.
- Choose the thermal cycler pre-heat lid option and set to 30°C
- Apply a ALP barcode label to a new 96-well 0.3 ml PCR plate.

Add SMM

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

6 Return the Second Strand Marking Master Mix tube to -15° to -25°C storage after use.

Incubate 2 CDP

1 Place the sealed CDP plate on the pre-heated thermal cycler. Close the lid and incubate at 16°C for 1 hour.

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

Purify CDP

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

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

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

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

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

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.

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

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

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

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

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

SAFE STOPPING POINT

If you do not plan to proceed to *Adenylate 3′ Ends* on page 74 immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Adenylate 3' Ends

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

Consumables

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

Preparation

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

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

  - A-Tailing Mix

- Remove the Resuspension Buffer from 2° to 8°C storage and bring it to room temperature.
- Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Purify CDP* on page 72 and let stand to thaw at room temperature.
  - Centrifuge the thawed ALP plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the ALP plate.
- Pre-program the thermal cycler with the following program and save as **ATAIL70**:
  - Choose the pre-heat lid option and set to 100°C
  - 37°C for 30 minutes
  - 70°C for 5 minutes
  - Hold at 4°C

Add ATL

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

Incubate 1 ALP

1. Place the sealed ALP plate on the pre-programmed thermal cycler. Close the lid and select **ATAIL70**.
2. When the thermal cycler temperature is 4°C, remove the ALP plate from the thermal cycler, then proceed immediately to *Ligate Adapters* on page 76.

Ligate Adapters

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

Consumables

Item	Quantity	Storage	Supplied By
(Optional) Ligation Control (CTL)	1 tube per 48 reactions	-15° to -25°C	Illumina

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

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

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

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - Appropriate RNA Adapter tubes (depending on the RNA Adapter Indices being used) or the RAP.
    - If using the RAP, review *Handling Adapter Plate* on page 44.
  - Stop Ligation Buffer

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

  - Ligation Control

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

- Remove the Resuspension Buffer from 2° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 10.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the thermal cycler to 30°C.
- Choose the thermal cycler pre-heat lid option and set to 100°C
- Apply a CAP barcode label to a new 96-well 0.3 ml PCR plate.
- Apply a PCR barcode label to a new 96-well 0.3 ml PCR plate.

NOTE

- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
- When indexing libraries with the RAP, arrange samples that will be pooled together in the same orientation as the indices in the RAP.

NOTE

Illumina recommends that the RAP does not undergo more than 4 freeze-thaw cycles. To maximize the use of the RAP, process more than 24 samples at a time. These samples can then be pooled in any supported configuration.

Add LIG

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

- If this is the first time using this RAP, apply the RAP barcode label to the plate.

2 Centrifuge the Ligation Control (if using Ligation Control) and Stop Ligation Buffer tubes to 600 xg for 5 seconds.

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

4 Remove the adhesive seal from the ALP plate.

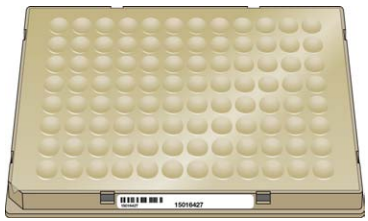
5 Do one of the following:
- If using the in-line control reagent:
  - Dilute the Ligation Control to 1/100 in Resuspension Buffer (For example, 1 µl Ligation Control + 99 µl Resuspension Buffer) before use. Discard the diluted Ligation Control after use.
  - Add 2.5 µl of diluted Ligation Control to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

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

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

8 Do one of the following:
- If using RNA Adapter tubes, add 2.5 µl of the appropriate/desired thawed RNA Adapter Index to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.
- If using a RAP:
  - Place the RAP on the benchtop so that the part number barcode on the long side of the plate is facing you and the clipped corner is located on the lower left.

Figure 38 Correct RAP Orientation



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

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

- Using an 8-tip multichannel pipette, transfer 2.5 µl of the appropriate/desired thawed RNA Adapter from the RAP well to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

10 Centrifuge the ALP plate to 280 xg for 1 minute.

Incubate 2 ALP

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

2 Remove the ALP plate from the thermal cycler.

Add STL

1 Remove the adhesive seal from the ALP plate.

2 Add 5 µl of Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation. Gently pipette the entire volume up and down 10 times to mix thoroughly.

Clean Up ALP

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

1 Vortex the AMPure XP Beads until they are well dispersed, then add 42 µl of mixed AMPure XP Beads to each well of the ALP plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

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

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

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

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

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

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

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

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

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

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

12 Transfer 50 µl of the clear supernatant from each well of the ALP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the CAP barcode.

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

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

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

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

NOTE

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

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

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

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

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

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

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

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

24 Transfer 20 µl of the clear supernatant from each well of the CAP plate to the corresponding well of the new 0.3 ml PCR plate labeled with the PCR barcode.

SAFE STOPPING POINT

If you do not plan to proceed to *Enrich DNA Fragments* on page 83 immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Enrich DNA Fragments

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

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

Consumables

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

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

Preparation

- Remove the PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature.
- Centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- Remove the Resuspension Buffer from 2° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 10.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the PCR plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 80 and let stand to thaw at room temperature.
  - Centrifuge the thawed PCR plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed PCR plate.
- Pre-program the thermal cycler with the following program and save as **PCR**:
  - Choose the pre-heat lid option and set to 100°C
  - 98°C for 30 seconds
  - 15 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 4°C
- Apply a TSP1 barcode label to a new 96-well 0.3 ml PCR plate.

Make PCR

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

Amp PCR

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

Clean Up PCR

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

1. Remove the adhesive seal from the PCR plate.
2. Vortex the AMPure XP Beads until they are well dispersed, then do one of the following:
   - If using the RNA Adapter tubes, add 50 µl of the mixed AMPure XP Beads to each well of the PCR plate containing 50 µl of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.
   - If using the RAP, add 47.5 µl of the mixed AMPure XP Beads to each well of the PCR plate containing 50 µl of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.
3. Incubate the PCR plate at room temperature for 15 minutes.
4. Place the PCR plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.
5. Remove and discard 95 µl of the supernatant from each well of the PCR plate.

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

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

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

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

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

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

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

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

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

SAFE STOPPING POINT

If you do not plan to proceed to *Validate Library* on page 87 immediately, the protocol can be safely stopped here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Validate Library

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

Quantify Libraries

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

Quality Control

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

Figure 39 Example of TruSeq Stranded mRNA Sample Prep Library Size Distribution

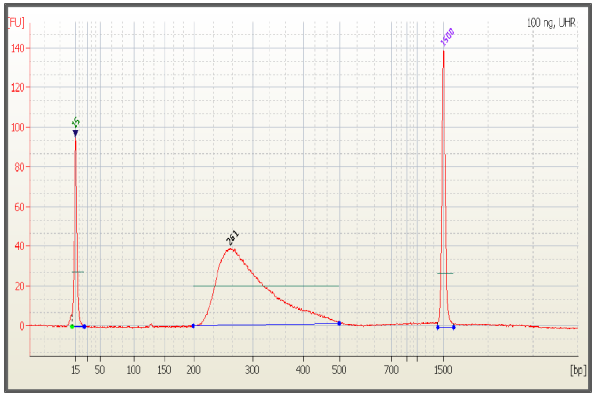
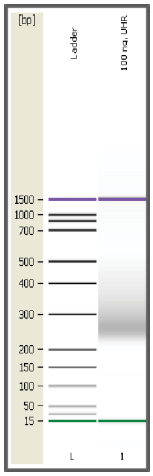


Figure 40 TruSeq Stranded mRNA Sample Prep 260 bp PCR Product



Normalize and Pool Libraries

This process describes how to prepare DNA templates that will be applied to cluster generation. Indexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. DNA libraries not intended for indexing are normalized to 10 nM in the DCT plate without pooling.

Consumables

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

Preparation

- Apply a DCT barcode label to a new 96-well MIDI plate.
- Apply a PDP barcode label to a new 96-well 0.3 ml PCR plate (for indexing only).
- Remove the TSP1 plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 85, and let stand to thaw at room temperature.
  - Centrifuge the thawed TSP1 plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.

Make DCT

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

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

Make PDP (for indexing only)

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

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

   NOTE
   Keep track of which sample goes into which well, to avoid pooling two samples with the same index.
2. Do one of the following:
   - If pooling 2–24 samples:
     - Transfer 10 µl of each normalized sample library to be pooled from the DCT plate to one well of the new 0.3 ml PCR plate labeled with the PDP barcode.

The total volume in each well of the PDP plate should be 10X the number of combined sample libraries and will be 20–240 µl (2–24 libraries). For example, the volume for 2 samples is 20 µl, the volume for 12 samples is 120 µl, or the volume for 24 samples is 240 µl.

- If pooling 25–96 samples:
  - Using a multichannel pipette, transfer 5 µl of each normalized sample library in column 1 from the DCT plate to column 1 of the new MIDI plate labeled with the PDP barcode.
  - Transfer 5 µl of each normalized sample library in column 2 from the DCT plate to column 1 of the PDP plate.
  - Repeat the transfer for as many times as there are remaining columns in the DCT plate. The result will be a PDP plate with pooled samples in column 1. Gently pipette the entire volume of each well of column 1 up and down 10 times to mix thoroughly.
  - Combine the contents of each well of column 1 into well A2 of the PDP plate, for the final pool.

3 Gently pipette the entire volume up and down 10 times to mix thoroughly.

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

High Sample (HS) Protocol

Chapter 4

Introduction

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

Table 19 Kit and Sample Number Recommendations

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

Table 20 Kit and Protocol Recommendations

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

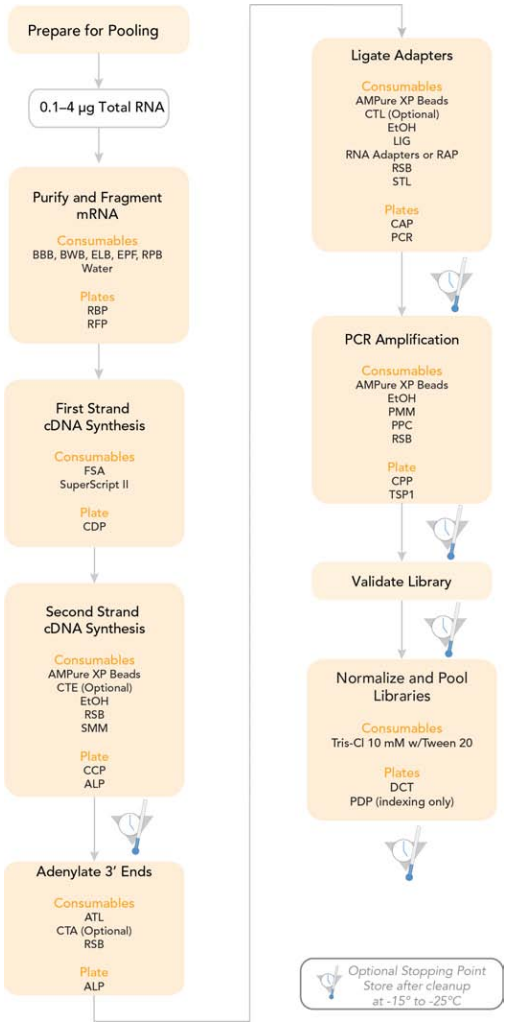
- Review *Best Practices* on page 9 before proceeding.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- This HS protocol requires shaking and heating equipment to mix reagents and for incubation (see *Consumables and Equipment* on page 35).

- For optimal sample tracking and quality control, fill out the Lab Tracking Form as you perform the sample preparation. For more information, see *Tracking Tools* on page 25.
- If you are pooling using adapter index tubes, record information about your samples before beginning library preparation for later use in data analysis. For more information, see *Tracking Tools* on page 25. Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
- If you are pooling with the RAP, review the planning steps in *Pooling Preparation with Adapter Plate* on page 43 before beginning library preparation.

Sample Prep Workflow

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

Figure 41 TruSeq Stranded mRNA Sample Preparation HS Workflow

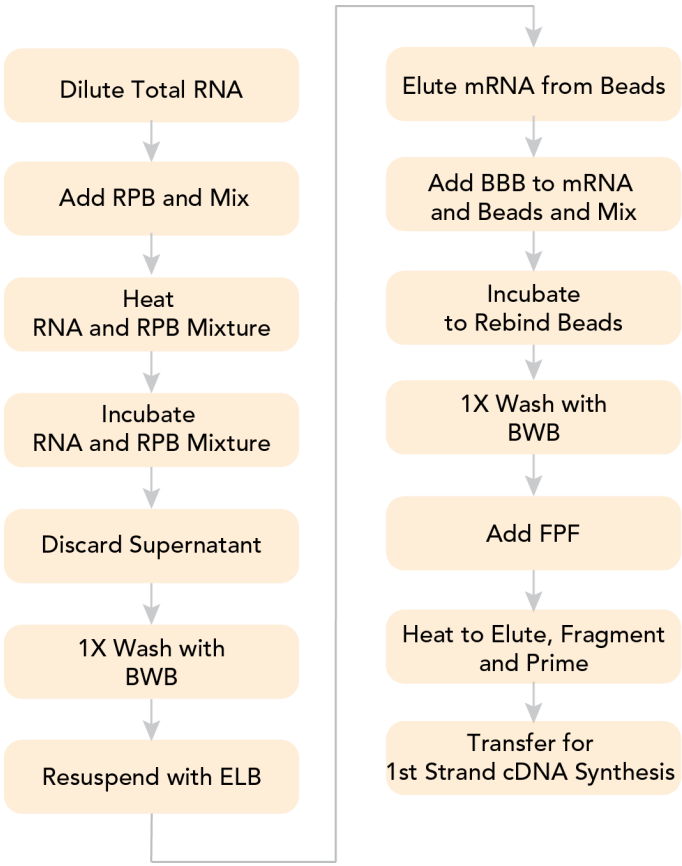


Purify and Fragment mRNA

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 42 TruSeq Stranded mRNA Sample Prep Purification Workflow



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

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

NOTE
Illumina recommends that you use 0.1–4 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, the mRNA must be concentrated into 5 µl or less before addition to Fragment, Prime, Finish Mix. This can be done by ethanol precipitation or by concentration on a Qiagen MinElute column.

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

In either case, heat the mRNA in Elute, Prime, Fragment Mix to fragment at *Incubate RFP* on page 103 in this process.

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

Consumables

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

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	-15° to -25°C	Illumina
RNA Purification Beads (RPB)	1 tube per 48 reactions	2° to 8°C	Illumina
RBP (RNA Bead Plate) barcode label	1 label per plate	15° to 30°C	Illumina
RFP (RNA Fragmentation Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well HSP plate	1	15° to 30°C	User
96-well MIDI plate	1	15° to 30°C	User
Microseal 'B' Adhesive Seals	7	15° to 30°C	User
RNase/DNase-free Reagent Reservoirs (if using multichannel pipettes)	6	15° to 30°C	User
RNase/DNase-free Strip Tubes and Caps (if using multichannel pipettes)	6	15° to 30°C	User

Preparation

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

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

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

- Pre-heat the microheating system to 65°C.
- Pre-program the thermal cycler with the following program and save as **Elution 2 - Frag - Prime**:
  - Choose the pre-heat lid option and set to 100°C
  - 94°C for 8 minutes
  - Hold at 4°C
- Make sure that the microplate shaker is properly calibrated to 1,000 rpm using a stroboscope.
- Set the centrifuge to 15° to 25°C, if refrigerated.
- Apply a RBP barcode label to a new 96-well MIDI plate.
- Apply a RFP barcode label to a new 96-well HSP plate.

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

Make RBP

1. Dilute the total RNA with nuclease-free ultra pure water to a final volume of 50 µl in the new 96-well MIDI plate labeled with the RBP barcode.
2. Vortex the room temperature RNA Purification Beads tube vigorously to completely resuspend the oligo-dT beads.
3. Add 50 µl of RNA Purification Beads to each well of the RBP plate to bind the poly-A RNA to the oligo dT magnetic beads. Mix thoroughly as follows:
   a. Seal the RBP plate with a Microseal 'B' Adhesive seal.
   b. Shake the RBP plate on a microplate shaker continuously at 1,000 rpm for 1 minute.

Incubate 1 RBP

1. Place the sealed RBP plate on the pre-heated microheating system. Close the lid and incubate at 65°C for 5 minutes 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 Remove the adhesive seal from the RBP plate.

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

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

4 Remove the RBP plate from the magnetic stand.

5 Wash the beads by adding 200 µl of Bead Washing Buffer in each well of the RBP plate to remove unbound RNA. Mix thoroughly as follows:
   a Seal the RBP plate with a Microseal 'B' Adhesive seal.
   b Shake the RBP plate on a microplate shaker continuously at 1,000 rpm for 1 minute.

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

7 Remove the adhesive seal from the RBP plate.

8 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. The supernatant contains the majority of the ribosomal and other non-messenger RNA.

10 Remove the RBP plate from the magnetic stand.

11 Add 50 µl of Elution Buffer in each well of the RBP plate. 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. Close the lid and incubate at 80°C for 2 minutes to elute the mRNA from the beads. This releases both the mRNA and any contaminant rRNA that has bound the beads non-specifically.
2. Remove the RBP plate from the microheating system and place on ice for 1 minute.
3. Place the RBP plate on the bench at room temperature and remove the adhesive seal from the plate.

Make RFP

1. Centrifuge the thawed Bead Binding Buffer to 600 xg for 5 seconds.
2. Add 50 µl of Bead Binding Buffer to each well of the RBP plate. This allows mRNA to specifically rebind the beads, while reducing the amount of rRNA that non-specifically binds. 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.
3. Incubate the RBP plate at room temperature for 5 minutes and store the Bead Binding Buffer tube at 2° to 8°C.
4. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.
5. Remove the adhesive seal from the RBP plate.
6. Remove and discard all of the supernatant from each well of the RBP plate.
7. Remove the RBP plate from the magnetic stand.
8. Wash the beads by adding 200 µl of Bead Washing Buffer in each well of the RBP plate. Mix thoroughly as follows:
   a. Seal the RBP plate with a Microseal 'B' Adhesive seal.
   b. Shake the RBP plate on a microplate shaker continuously at 1,000 rpm for 1 minute.
9. Store the Bead Washing Buffer tube at 2° to 8°C.
10. Place the RBP plate on the magnetic stand at room temperature for 5 minutes.

11 Remove the adhesive seal from the RBP plate.

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

13 Remove the RBP plate from the magnetic stand.

14 Add 19.5 µl of Fragment, Prime, Finish Mix to each well of the RBP plate. 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.

15 Remove the adhesive seal from the RBP plate.

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

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

18 Store the Fragment, Prime, Finish 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** 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 Second Strand cDNA* on page 107.

Synthesize First Strand cDNA

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

Consumables

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

WARNING

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

Preparation

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

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

Make CDP

1. Place the RFP plate on the magnetic stand at room temperature for 5 minutes. Do not remove the plate from the magnetic stand.
2. Remove the adhesive seal from the RFP plate.
3. Transfer 17 µl of the supernatant (fragmented and primed mRNA) from each well of the RFP plate to the corresponding well of the new HSP plate labeled with the CDP barcode.
4. Centrifuge the thawed First Strand Synthesis Act D Mix tube to 600 xg for 5 seconds.
5. Add 50 µl SuperScript II to the First Strand Synthesis Act D Mix tube. Mix gently, but thoroughly and centrifuge briefly. If you are not using the entire contents of the First Strand Synthesis Act D Mix tube, add SuperScript II at a ratio of 1 µl SuperScript II for each 9 µl First Strand Synthesis Act D Mix.

Label the First Strand Synthesis Act D Mix tube to indicate that the SuperScript II has been added.

6 Add 8 µl of First Strand Synthesis Act D Mix and SuperScript II mix to each well of the CDP plate. Mix thoroughly as follows:
   a Seal the CDP plate with a Microseal 'B' Adhesive seal.
   b Shake the CDP plate on a microplate shaker continuously at 1,600 rpm for 20 seconds.

7 Return the First Strand Synthesis Act D Mix tube to -15° to -25°C storage immediately after use.

Incubate 1 CDP

1 Place the sealed CDP plate on the pre-programmed thermal cycler. Close the lid and select the **Synthesize 1st Strand** program.

2 When the thermal cycler reaches 4°C, remove the CDP plate from the thermal cycler and proceed immediately to *Synthesize Second Strand cDNA* on page 107.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand, incorporating dUTP in place of dTTP to generate ds cDNA. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix. At the end of this process, you will have blunt-ended cDNA.

Consumables

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

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

Preparation

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

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

  - Second Strand Marking Master Mix
- Remove the Resuspension Buffer from 2° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 10.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the thermal cycler to 16°C.
- Choose the thermal cycler pre-heat lid option and set to 30°C
- Apply a ALP barcode label to a new 96-well MIDI plate.
- Apply a CCP barcode label to a new 96-well MIDI plate.

Add SMM

1. Remove the adhesive seal from the CDP plate.
2. Do one of the following:
   - If using the in-line control reagent:
     - Centrifuge the thawed End Repair Control tube to 600 xg for 5 seconds.
     - Dilute the End Repair Control to 1/50 in Resuspension Buffer (For example, 2 µl End Repair Control + 98 µl Resuspension Buffer) before use. Discard the diluted End Repair Control after use.
     - Add 5 µl of diluted End Repair Control to each well of the CDP plate.

- If not using the in-line control reagent, add 5 µl of Resuspension Buffer to each well of the CDP plate.

3. Centrifuge the thawed Second Strand Marking Master Mix to 600 xg for 5 seconds.
4. Add 20 µl of thawed Second Strand Marking Master Mix to each well of the CDP plate. Mix thoroughly as follows:
   a. Seal the CDP plate with a Microseal 'B' Adhesive seal.
   b. Shake the CDP plate on a microplate shaker continuously at 1,600 rpm for 20 seconds.
5. Return the Second Strand Marking Master Mix tube to -15° to -25°C storage after use.

Incubate 2 CDP

1. Place the sealed CDP plate on the pre-heated thermal cycler. Close the lid and incubate at 16°C for 1 hour.
2. Remove the CDP plate from the thermal cycler, remove the adhesive seal, and let stand to bring the plate to room temperature.

Purify CDP

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

1. Vortex the AMPure XP beads until they are well dispersed, then add 90 µl of well-mixed AMPure XP beads to each well of the new MIDI plate labeled with the CCP barcode.
2. Transfer the entire contents from each well of the CDP plate to the corresponding well of the CCP plate containing AMPure XP beads. Mix thoroughly as follows:
   a. Seal the CCP plate with a Microseal 'B' Adhesive seal.
   b. Shake the CCP plate on a microplate shaker at 1,800 rpm for 2 minutes.
3. Incubate the CCP plate at room temperature for 15 minutes.
4. Place the CCP plate on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells.

5 Remove the adhesive seal from the CCP plate.

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

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

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

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

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

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

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

12 Add 17.5 µl Resuspension Buffer to each well of the CCP plate. Mix thoroughly as follows:

a Seal the CCP plate with a Microseal 'B' Adhesive seal.

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

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

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

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

15 Remove the adhesive seal from the CCP plate.

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

SAFE STOPPING POINT
If you do not plan to proceed to *Adenylate 3' Ends* on page 111 immediately, the protocol can be safely stopped here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Adenylate 3' Ends

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

Consumables

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

Preparation

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

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

  - A-Tailing Mix

- Remove the Resuspension Buffer from 2° to 8°C storage and bring it to room temperature.
- Remove the ALP plate from -15° to -25°C storage, if it was stored at the conclusion of *Purify CDP* on page 109 and let stand to thaw at room temperature.
  - Centrifuge the thawed ALP plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the ALP plate.
- Pre-heat two microheating systems: system 1 to 37°C and system 2 to 70°C.

Add ATL

1. Do one of the following:
   - If using the in-line control reagent:
     - Centrifuge the thawed A-Tailing Control tube to 600 xg for 5 seconds.
     - Dilute the A-Tailing Control to 1/100 in Resuspension Buffer (For example, 1 µl A-Tailing Control + 99 µl Resuspension Buffer) before use. Discard the diluted A-Tailing Control after use.
     - Add 2.5 µl of diluted A-Tailing Control to each well of the ALP plate.
   - If not using the in-line control reagent, add 2.5 µl of Resuspension Buffer to each well of the ALP plate.
2. Add 12.5 µl of thawed A-Tailing Mix to each well of the ALP plate. Mix thoroughly as follows:
   a. Seal the ALP plate with a Microseal 'B' adhesive seal.
   b. Shake the ALP plate on a microplate shaker at 1,800 rpm for 2 minutes.
   c. Centrifuge the ALP plate to 280 xg for 1 minute.

Incubate 1 ALP

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

4 Immediately remove the ALP plate from the microheating system 2 and place the plate on ice for 1 minute.

5 Proceed immediately to *Ligate Adapters* on page 114.

Ligate Adapters

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

Consumables

Item	Quantity	Storage	Supplied By
(Optional) Ligation Control (CTL)	1 tube per 48 reactions	-15° to -25°C	Illumina

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

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

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

Preparation

- Remove the following from -15° to -25°C storage and thaw them at room temperature:
  - Appropriate RNA Adapter tubes (depending on the RNA Adapter Indices being used) or the RAP.
    - If using the RAP, review *Handling Adapter Plate* on page 44.
  - Stop Ligation Buffer

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

  - Ligation Control

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

- Remove the Resuspension Buffer from 2° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 10.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-heat the microheating system 1 to 30°C.
- Apply a CAP barcode label to a new 96-well MIDI plate.
- Apply a PCR barcode label to a new 96-well HSP plate.

NOTE

- When indexing libraries using adapter index tubes, Illumina recommends arranging samples that will be combined into a common pool in the same row. Each column should contain a common index. This will facilitate pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.
- When indexing libraries with the RAP, arrange samples that will be pooled together in the same orientation as the indices in the RAP.

NOTE

Illumina recommends that the RAP does not undergo more than 4 freeze-thaw cycles. To maximize the use of the RAP, process more than 24 samples at a time. These samples can then be pooled in any supported configuration.

Add LIG

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

2 Centrifuge the Ligation Control (if using Ligation Control) and Stop Ligation Buffer tubes to 600 xg for 5 seconds.

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

4 Remove the adhesive seal from the ALP plate.

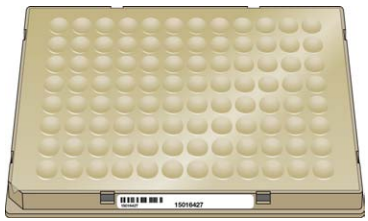
5 Do one of the following:
- If using the in-line control reagent:
  - Dilute the Ligation Control to 1/100 in Resuspension Buffer (For example, 1 µl Ligation Control + 99 µl Resuspension Buffer) before use. Discard the diluted Ligation Control after use.
  - Add 2.5 µl of diluted Ligation Control to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

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

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

8 Do one of the following:
- If using RNA Adapter tubes, add 2.5 µl of the appropriate/desired thawed RNA Adapter Index to each well of the ALP plate.
- If using a RAP:
  - Place the RAP on the benchtop so that the part number barcode on the long side of the plate is facing you and the clipped corner is located on the lower left.

Figure 43 Correct RAP Orientation



  - Do one of the following to pierce the foil seal:
    - If using the entire plate at once, use the bottom of a clean 96-well semi-skirted PCR plate to pierce a hole in all of the well seals simultaneously by gently but firmly pressing the clean plate over the foil seal.

- If using only part of the plate, use the bottom of a clean eight-tube strip, with caps attached, to pierce holes in the desired columns that will be used for ligation. Repeat with a new, clean eight-tube strip, with caps attached, for each column of adapters that will be used for ligation.
- Using an 8-tip multichannel pipette, transfer 2.5 µl of the appropriate/desired thawed RNA Adapter from the RAP well to each well of the ALP plate.

9 Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1,800 rpm for 2 minutes.
   c Centrifuge the ALP plate to 280 xg for 1 minute.

Incubate 2 ALP

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

2 Remove the ALP plate from the microheating system.

Add STL

1 Remove the adhesive seal from the ALP plate.

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

Clean Up ALP

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

1 Remove the adhesive seal from the ALP plate.

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

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

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

5 Remove the adhesive seal from the ALP plate.

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

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

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

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

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

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

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

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

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

14 Remove the adhesive seal from the ALP plate.

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

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

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

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

19 Remove the adhesive seal from the CAP plate.

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

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

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

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

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

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

25 Resuspend the dried pellet in each well with 22.5 µl Resuspension Buffer. Mix thoroughly as follows:
   a Seal the CAP plate with a Microseal 'B' adhesive seal.
   b Shake the CAP plate on a microplate shaker at 1,800 rpm for 2 minutes.

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

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

28 Remove the adhesive seal from the CAP plate.

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

SAFE STOPPING POINT

If you do not plan to proceed to *Enrich DNA Fragments* on page 122 immediately, the protocol can be safely stopped here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Enrich DNA Fragments

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

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

Consumables

Item	Quantity	Storage	Supplied By
PCR Master Mix (PMM)	1 tube per 48 reactions	-15° to -25°C	Illumina
PCR Primer Cocktail (PPC)	1 tube per 48 reactions	-15° to -25°C	Illumina
Resuspension Buffer (RSB)	1 tube	2° to 8°C	Illumina
CPP (Clean Up PCR Plate) barcode label	1 label per plate	15° to 30°C	Illumina
TSP1 (Target Sample Plate) barcode label	1 label per plate	15° to 30°C	Illumina
96-well HSP plate	1	15° to 30°C	User
96-well MIDI plate	1	15° to 30°C	User
AMPure XP beads	50 µl per sample	2° to 8°C	User

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

Preparation

- Remove the PCR Master Mix and PCR Primer Cocktail from -15° to -25°C storage and thaw them at room temperature.
- Centrifuge the thawed PCR Master Mix and PCR Primer Cocktail tubes to 600 xg for 5 seconds.
- Remove the Resuspension Buffer from 2° to 8°C storage and bring it to room temperature.
- Review *Handling Magnetic Beads* on page 10.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the PCR plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 118 and let stand to thaw at room temperature.
  - Centrifuge the thawed PCR plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed PCR plate.
- Pre-program the thermal cycler with the following program and save as **PCR**:
  - Choose the pre-heat lid option and set to 100°C
  - 98°C for 30 seconds
  - 15 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds

  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°C

▶ Apply a CPP barcode label to a new 96-well MIDI plate.

▶ Apply a TSP1 barcode label to a new 96-well HSP plate.

Make PCR

1 Add 5 µl of thawed PCR Primer Cocktail to each well of the PCR plate.

2 Add 25 µl of thawed PCR Master Mix to each well of the PCR plate. Mix thoroughly as follows:

a Seal the PCR plate with a Microseal 'A' film.

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

b Shake the PCR plate on a microplate shaker at 1,600 rpm for 20 seconds.

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

Amp PCR

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

Clean Up PCR

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

1 Remove the adhesive seal from the PCR plate.

2 Vortex the AMPure XP Beads until they are well dispersed, then do one of the following:

- If using the RNA Adapter tubes, add 50 µl of the mixed AMPure XP Beads to each well of the new MIDI plate labeled with the CPP barcode.

- If using the RAP, add 47.5 µl of the mixed AMPure XP Beads to each well of the new MIDI plate labeled with the CPP barcode.

3 Transfer the entire contents from each well of the PCR plate to the corresponding well of the CPP plate containing 50 µl of mixed AMPure XP Beads. Mix thoroughly as follows:
   a Seal the CPP plate with a Microseal 'B' adhesive seal.
   b Shake the CPP plate on a microplate shaker at 1,800 rpm for 2 minutes.

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

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

6 Remove the adhesive seal from the CPP plate.

7 Remove and discard 95 µl of the supernatant from each well of the CPP plate.

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

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

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

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

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

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

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

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

15 Remove the adhesive seal from the CPP plate.

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

SAFE STOPPING POINT

If you do not plan to proceed to *Validate Library* on page 127 immediately, the protocol can be safely stopped here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -15° to -25°C for up to seven days.

Validate Library

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

Quantify Libraries

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

Quality Control

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

Figure 44 Example of TruSeq Stranded mRNA Sample Prep Library Size Distribution

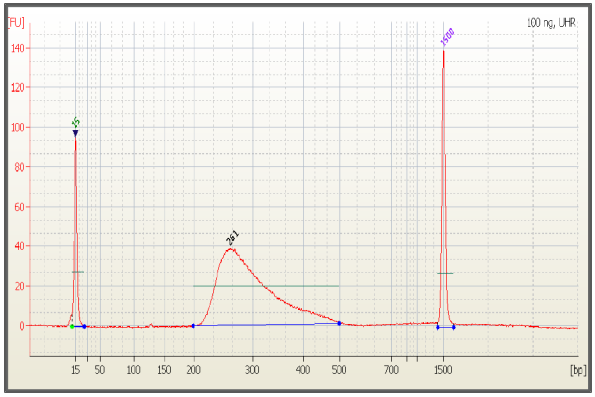
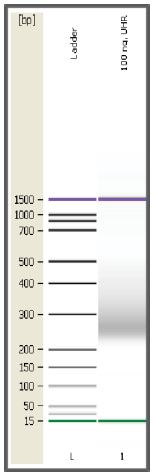


Figure 45 TruSeq Stranded mRNA Sample Prep 260 bp PCR Product



Normalize and Pool Libraries

This process describes how to prepare DNA templates that will be applied to cluster generation. Indexed DNA libraries are normalized to 10 nM in the DCT plate and then pooled in equal volumes in the PDP plate. DNA libraries not intended for indexing are normalized to 10 nM in the DCT plate without pooling.

Consumables

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

Preparation

- Apply a DCT barcode label to a new 96-well MIDI plate.
- Apply a PDP barcode label to a new 96-well HSP plate (for indexing only).
- Remove the TSP1 plate from -15° to -25°C storage, if it was stored at the conclusion of *Clean Up PCR* on page 124, and let stand to thaw at room temperature.
  - Centrifuge the thawed TSP1 plate to 280 xg for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.

Make DCT

1 Transfer 10 µl of sample library from each well of the TSP1 plate to the corresponding well of the new MIDI plate labeled with the DCT barcode.

2 Normalize the concentration of sample library in each well of DCT plate to 10 nM using Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20.

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

3 Mix the DCT plate as follows:

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

b Shake the DCT plate on a microplate shaker at 1,000 rpm for 2 minutes.

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

d Remove the adhesive seal from the DCT plate.

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

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

Make PDP (for indexing only)

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

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

NOTE
Keep track of which sample goes into which well, to avoid pooling two samples with the same index.

2 Do one of the following:

- If pooling 2–24 samples:

- Transfer 10 µl of each normalized sample library to be pooled from the DCT plate to one well of the new 0.3 ml PCR plate labeled with the PDP barcode.

The total volume in each well of the PDP plate should be 10X the number of combined sample libraries and will be 20–240 µl (2–24 libraries). For example, the volume for 2 samples is 20 µl, the volume for 12 samples is 120 µl, or the volume for 24 samples is 240 µl.

- If pooling 25–96 samples:
  - Using a multichannel pipette, transfer 5 µl of each normalized sample library in column 1 from the DCT plate to column 1 of the new MIDI labeled with the PDP barcode.
  - Transfer 5 µl of each normalized sample library in column 2 from the DCT plate to column 1 of the PDP plate.
  - Repeat the transfer for as many times as there are remaining columns in the DCT plate. The result will be a PDP plate with pooled samples in column 1. Mix the PDP plate as follows:
    - Seal PDP plate with Microseal 'B' adhesive seal.
    - Shake PDP plate on microplate shaker at 1,800 rpm for 2 minutes.
  - Combine the contents of each well of column 1 into well A2 of the PDP plate, for the final pool.

3. Mix the PDP plate as follows:
   a. Seal the PDP plate with a Microseal 'B' adhesive seal.
   b. Shake the PDP plate on a microplate shaker at 1,800 rpm for 2 minutes.
4. Do one of the following:
   - Proceed to cluster generation. For more information, see the *Illumina Cluster Generation User Guide*.
   - Seal the PDP plate with a Microseal 'B' adhesive seal and store at -15° to -25°C.

Alternate Fragmentation Protocols

Appendix A

Introduction

Fragmentation of the nucleic acids is required for optimal library preparation, clustering and sequencing. The TruSeq Stranded mRNA Sample Prep fragmentation protocol for transcriptome analysis is performed on the RNA after mRNA purification using elevated temperatures, resulting in libraries with inserts ranging in size from 120-200 bp with a median size of 150 bp. The TruSeq Stranded mRNA Sample Prep fragmentation protocol ensures the best coverage of the transcriptome with efficient library production.

Illumina recognizes that some customers have different purposes for their sequencing experiments. The need for larger inserts is greater than the need for the best coverage for applications such as splice variant analysis studies. Two separate options are provided for varying the insert size of your library:

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

Modify RNA Fragmentation Time

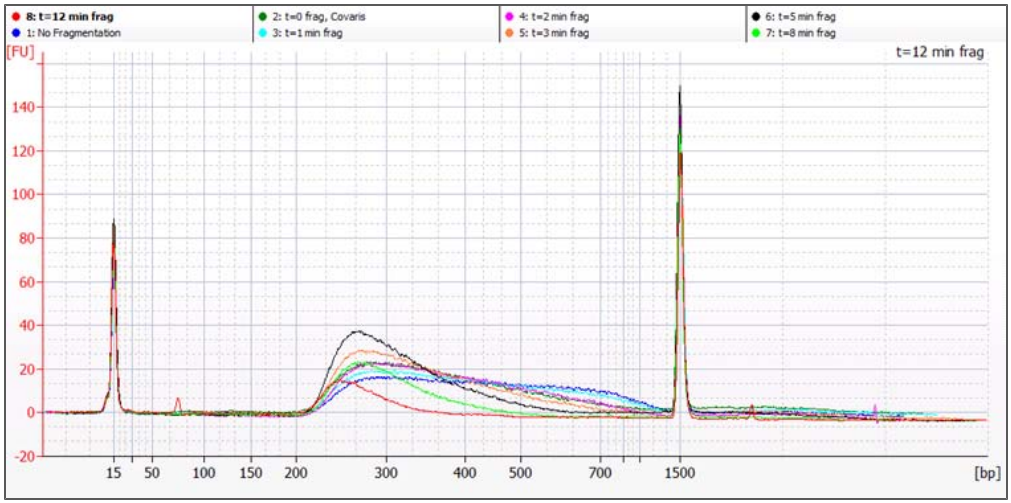
To modify the fragmentation of the RNA to allow for longer RNA fragments, the time of fragmentation can be shortened. This can be accomplished during the *Purify and Fragment mRNA* procedures by modifying the thermal cycler **Elution 2 - Frag - Prime** program: 94°C for X minutes followed by a 4°C hold for the thermal cycler where X is determined by the length of RNA desired. A range of suggested times and sizes is described in Table 21.

Table 21 Library Insert Fragmentation Time

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

a. Insert length determined after clustering and sequencing with a paired-end sequencing run.
b. Start without bringing up to temperature. Leave the sample at 4°C.

Figure 46 Shortened Fragmentation Time Results



NOTE

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

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Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 22 Illumina General Contact Information

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

Table 23 Illumina Customer Support Telephone Numbers

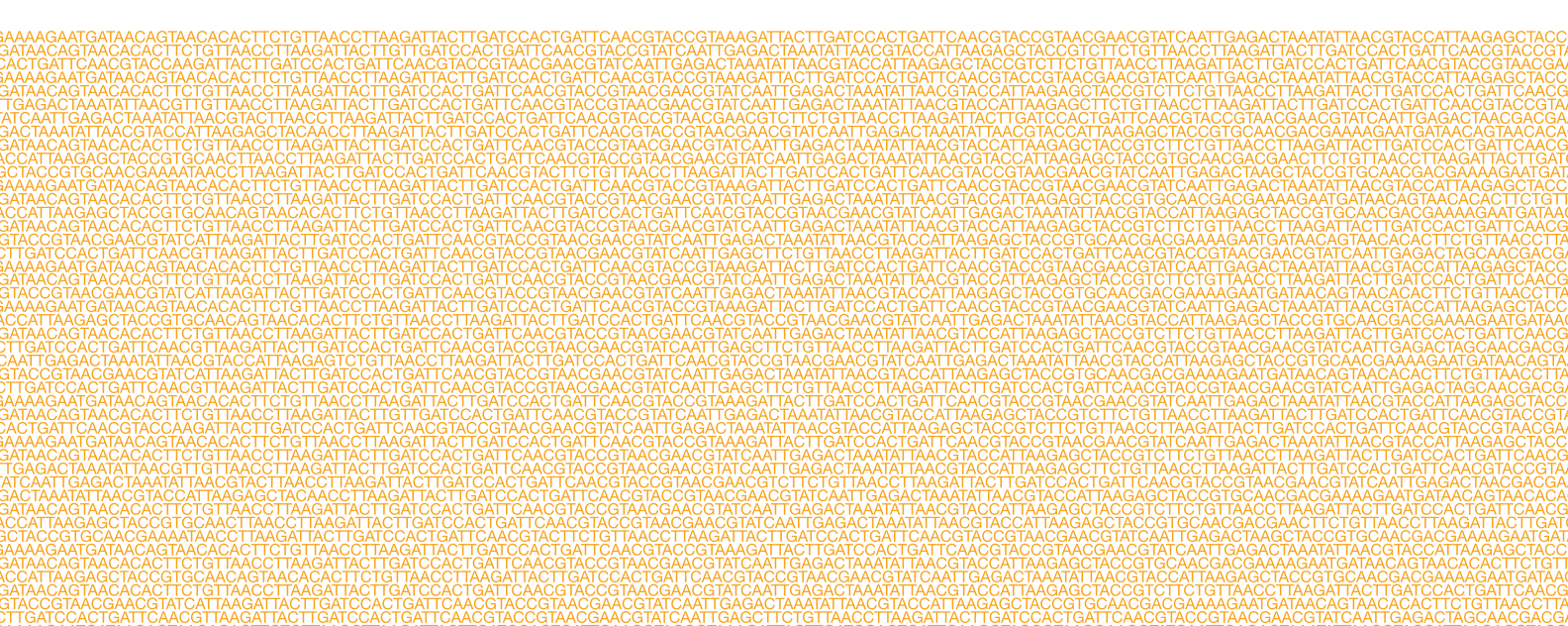
Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994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

MSDSs

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

Product Documentation

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



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