illumina®

TruSeq Stranded mRNA

Reference Guide

Document # 1000000040498 v00

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Chapter 1 Overview

Introduction

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

This library prep protocol offers:

- Strand information an RNA transcript
- Library capture of both coding RNA and multiple forms of noncoding RNA that are polyadenylated
- Optimized workflows for processing low sample (LS) and high sample (HS) numbers in parallel
- Inclusive components:
  - Library Prep components include library prep reagents excluding index adapters.
  - Index adapter components must be purchased separately. For more information, see *Supporting Information* on page 26.
  - The use of the included In-line Control DNA provided with this kit is optional and requires a custom analysis pipeline. If analysis is not available, omit them from the prep.

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 meet the yield requirements of most standard applications.

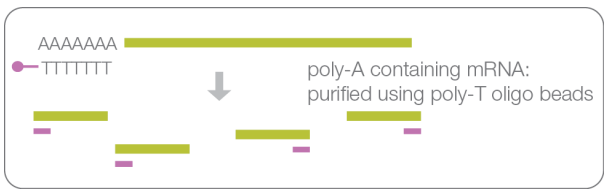
Process

The following workflow explains how the TruSeq Stranded mRNA Library Prep assay works, how strandedness is achieved, and which read maps to which strand.

Purify and Fragment mRNA

The Poly-A containing mRNA molecules are purified using poly-T oligo attached magnetic beads. Following purification, the mRNA is fragmented into small pieces using divalent cations under elevated temperature.

Figure 1 Purifying and Fragmenting mRNA



Synthesize First Strand cDNA

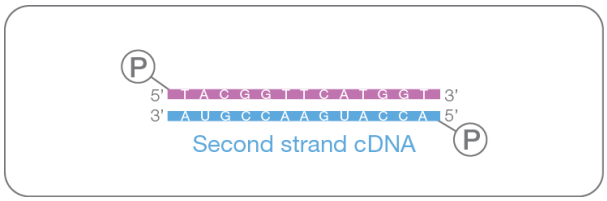
Cleaved RNA fragments are copied into first strand cDNA using reverse transcriptase and random primers. Adding Actinomycin D to FSA (First Stand Synthesis Act D mix) prevents spurious DNA-dependent synthesis, while allowing RNA-dependent synthesis, improving strand specificity.

Figure 2 Synthesizing First Strand cDNA

Synthesize Second Strand cDNA

Strand specificity is achieved by replacing dTTP with dUTP in the SMM (Second Strand Marking Mix), followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. The incorporation of dUTP in second strand synthesis quenches the second strand during amplification.

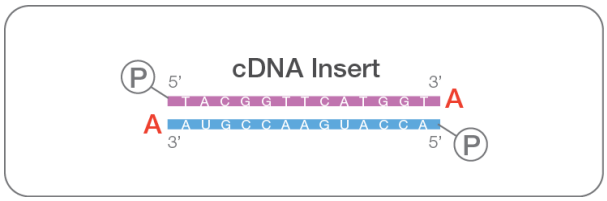
Figure 3 Synthesizing Second Strand cDNA



Adenylate 3' Ends

A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to each other 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.

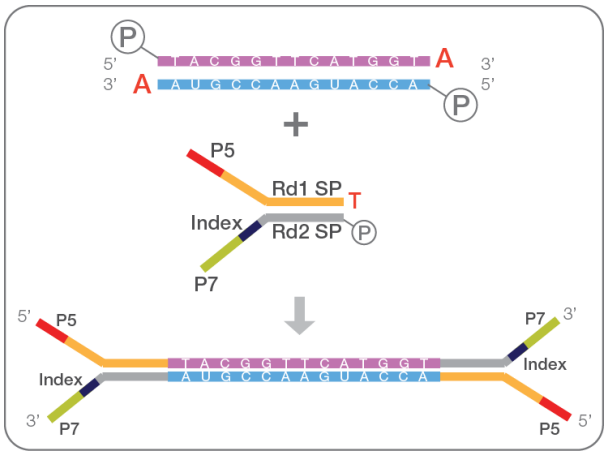
Figure 4 Adenylating 3' Ends



Ligate Adapters

The single-index adapter is shown in this workflow. The dual-index adapter option is not shown in this workflow. Adapter ligation prepares the ds cDNA for hybridization onto a flow cell.

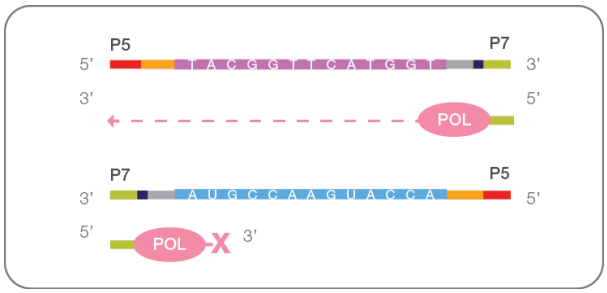
Figure 5 Ligating Adapters



Enrich DNA Fragments

Polymerase used in the assay does not incorporate past dUTP. Therefore, the second strand is effectively quenched during amplification. The products are enriched with PCR and purified to create the final cDNA library.

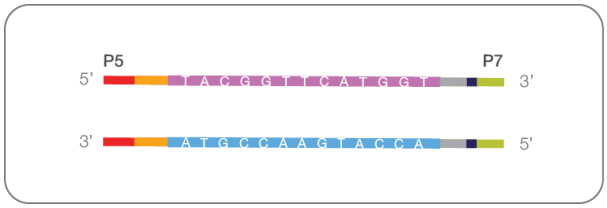
Figure 6 Enriching DNA Fragments



Final Library

The LS library features a single-index adapter, as shown in this workflow. The HS library features a dual-index adapter, which contains a unique index at each end. The HS library dual-index adapter is not shown in this workflow.

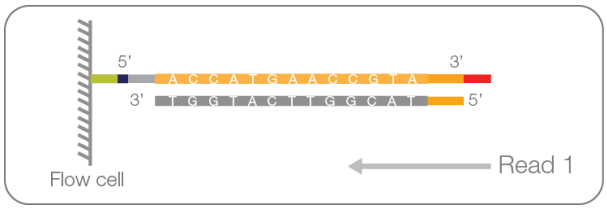
Figure 7 LS Final Library



Cluster Generation and Read 1 Sequencing

In Read 1, sequencing reads map to the antisense strand.

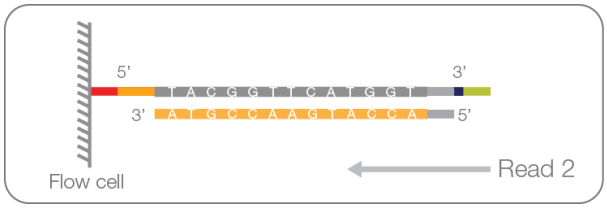
Figure 8 Cluster Generation and Read 1 Sequencing



Paired-end Turnaround and Read 2 Sequencing

In Read 2, sequencing reads map to the sense strand.

Figure 9 Paired-end Turnaround and Read 2 Sequencing



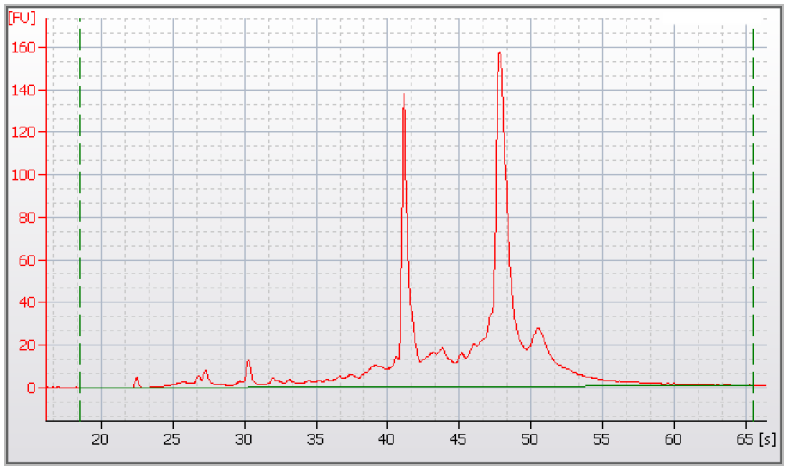
RNA Input Recommendations

Total RNA Input

- The protocol is optimized for 0.1–1 µg of total RNA.
  - Use a fluorometric method to quantify RNA.
  - Lower amounts can result in inefficient ligation and low yield.
- The protocol has been tested using 0.1–1 µg of high-quality universal human reference total RNA as input.
  - Use of RNA from other species, tissues, or lower quality RNA, including FFPE samples, might require further optimization to determine the input amount.
- Dilute inline controls for tracking the conversion of dsDNA into libraries.
  - The dilution is optimized for 0.1–1 µg of high-quality input RNA.
  - When using less RNA or RNA with very low mRNA content, the controls might need further dilution.
  - If controls are not used, use RSB (Resuspension Buffer) in their place in the protocol.
- Determine the quality of the RNA starting material. The fragmentation conditions are optimized for high-quality RNA.
  - Use an Agilent RNA 6000 Nano Kit or Advanced Analytical Standard Sensitivity RNA Analysis Kit to determine the quality of your starting material.
  - Do not use low quality or degraded RNA with this protocol. Use of degraded RNA can result in low yield, overrepresentation of the 3' ends of the RNA molecules, or failure of the protocol.
  - Check total RNA integrity following isolation:
    - On an Agilent Technologies 2100 Bioanalyzer, samples with an RNA Integrity Number (RIN) value ≥ 8, or on an Advanced Analytical Fragment Analyzer, samples with an RNA Quality Number (RQN) value ≥ 8, are recommended.
  - Using RNA with DNA contamination results in an overestimation of the amount of RNA used.

- Include a DNase step with the RNA isolation method to ensure purity and accurate quantification of the sample.
- The following figure shows a Universal Human Reference (UHR) starting RNA Bioanalyzer trace.

Figure 10 Starting RNA Bioanalyzer Trace



  - Alternatively, run a formaldehyde 1% agarose gel and determine the integrity of RNA upon staining with ethidium bromide.
    - High-quality RNA shows a 28S ribosomal RNA (rRNA) band at 4.5 kb with 2X the intensity of the 18S rRNA band at 1.9 kb.
    - Both kb determinations are relative to an RNA 6000 ladder.
    - The mRNA appears as a smear from 0.5–12 kb.

Purified mRNA Input

You can use 10–100 ng previously isolated mRNA as starting material. Use the entire fraction of mRNA purified from 0.1–1 µg of total RNA.

1 Concentrate the mRNA to ≤ 5 µl by ethanol precipitation or use a QIAGEN MinElute column before adding FPF (Fragment, Prime, Finish Mix).
   - If ethanol precipitation is used, resuspend the pellet in 18 µl FPF.
   - If a QIAGEN MinElute column is used, elute the mRNA with 5 µl molecular biology-grade water and add 13 µl FPF. Using a MinElute column results in a loss of up to 50% of the mRNA due to the low elution volume.

2 Proceed to *Fragment mRNA* on page 12 step 14.

Positive Control

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

Additional Resources

The following documentation is available for download from the Illumina website.

Resource	Description
Custom Protocol Selector	support.illumina.com/custom-protocol-selector.html A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
TruSeq Stranded mRNA Checklist (document # 10000000xxxxx	Provides a checklist of the protocol steps. The checklist is intended for experienced users.
Index Adpater Pooling Guide (document # 1000000041074)	Provides pooling guidelines for preparing libraries for Illumina sequencing systems that require balanced index combinations. Review this guide before beginning library preparation.
Sequencing Library qPCR Quantification Guide (document # 11322363)	Describes a qPCR method for quantifying sequencing by synthesis (SBS) libraries generated using the Illumina library prep protocols.
Illumina Experiment Manager Guide (document # 15031335) and *IEM TruSeq DNA, RNA, or ChIP Quick Reference Card (document # 15037152)*	Provide information about creating and editing appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate.
BaseSpace help (help.basespace.illumina.com)	Provides information about the BaseSpace® sequencing data analysis tool that also enables you to organize samples, libraries, pools, and sequencing runs in a single environment.
Local Run Manager Software Guide (document #100000002701)	Provides an overview of the Local Run Manager (LRM) software, instructions for using software features, and instructions for installing analysis modules on the instrument computer.

Visit the TruSeq Stranded mRNA workflow support page on the Illumina website for access to requirements and compatibility, additional documentation, software downloads, online training, frequently asked questions, and best practices.

Chapter 2 Protocol

Introduction

- Perform the protocol in the order described using specified volumes and incubation parameters.
- The protocol provides a single workflow with options depending on the number of samples processed.
  - Differences for each option are designated with [HS] or [LS].
  - Follow the instructions for the workflow option that supports your number of samples.
  - You can expect equivalent results from either option. However, the [HS] option can yield more consistent results between samples.
- Each option includes the following features.

Table 1 Workflow Variations

Workflow Variable	HS	LS
48 sample workflow	> 48 with index adapter tubes	≤ 48 with index adapter tubes
96 sample workflow	> 24 with index adapter plate	≤ 24 with index adapter plate
Plate Type	96-well Hard-Shell PCR plate 96-well midi plate	96-well 0.3 ml PCR plate 96-well midi plate
Incubation Equipment	96-well thermal cycler Microheating system	96-well thermal cycler
Mixing Method	Microplate shaker	Pipetting

- Review Best Practices before proceeding. See *Additional Resources* on page 6 for information on how to access TruSeq Stranded mRNA Library Prep Best Practices on the Illumina website.
- Before proceeding, confirm workflow contents and make sure that you have the required equipment and consumables. For more information, see *Supporting Information* on page 26.

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each row*** and ***each column***.
- Remove unused index adapter tubes from the working area.

Sealing the Plate

- Always seal the 96-well plate before the following steps in the protocol:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps
- Apply the adhesive seal to cover the plate, and seal with a rubber roller.
- Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' for shaking, centrifuging, and long-term storage.
- Microseal 'A' adhesive film is used for thermal cycling steps to prevent evaporation.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.

Centrifugation

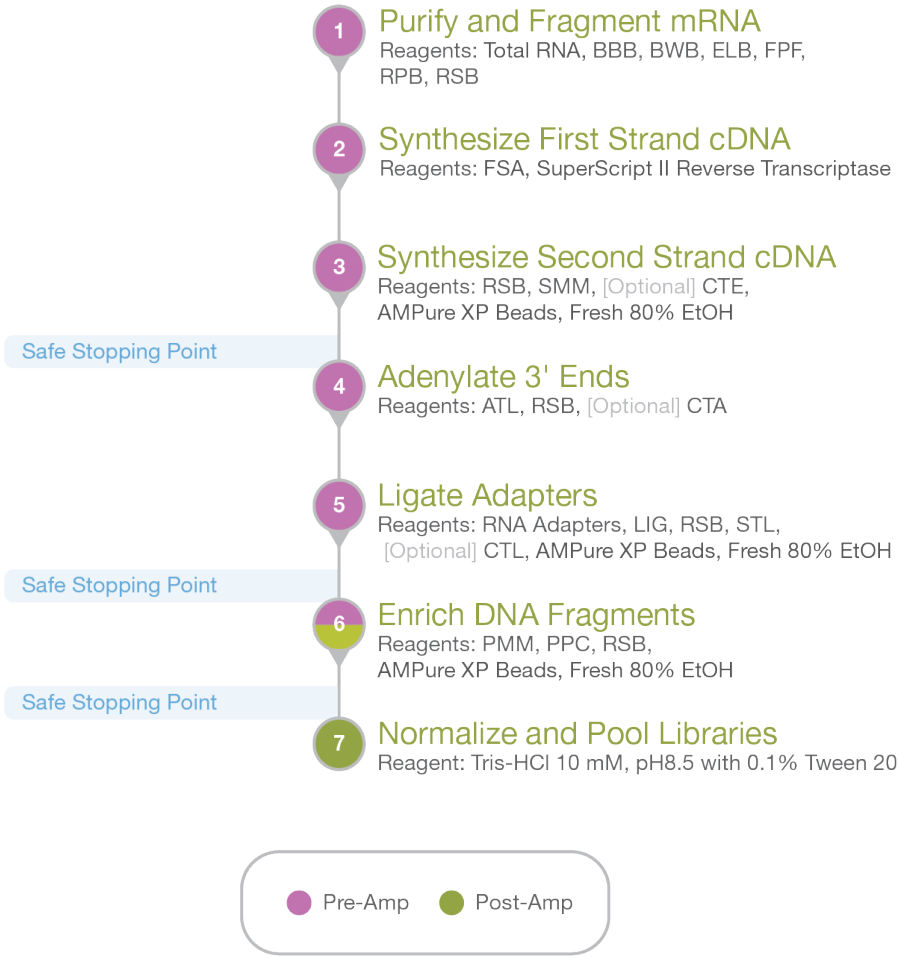
- Centrifuge at any step in the procedure to consolidate liquid or beads in the bottom of the well, and to prevent sample loss.

Handling Beads

- Do not freeze beads.
- Pipette bead suspensions slowly.
- Before use, allow the beads to come to room temperature.
- Immediately before use, vortex the beads until they are well dispersed. The color of the liquid must appear homogeneous. Vortex throughout protocol as necessary to keep homogenous.
- If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).
- When washing beads:
  - Use the specified magnetic stand for the plate.
  - Dispense liquid so that beads on the side of the wells are wetted.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - Do not agitate the plate while it is on the magnetic stand. Do not disturb the bead pellet.

Library Prep Workflow

Figure 11 TruSeq Stranded mRNA Library Prep Workflow



Prepare for Pooling

When pooling samples for sequencing, use IEM, LRM, or BaseSpace Prep Tab to record information about your samples before beginning library preparation.

- Use IEM to create and edit sample sheets for Illumina sequencing systems and analysis software.
- Use LRM and BaseSpace Prep Tab to organize samples, libraries, pools, and a run for Illumina sequencing systems and analysis software.

Review the planning steps in the *Index Adpater Pooling Guide (document # 1000000041074)* and the *Library pooling guidelines for the NextSeq and MiniSeq systems* technical bulletin when preparing libraries for Illumina sequencing systems that require balanced index combinations.

Purify and Fragment mRNA

This process purifies the polyA containing mRNA molecules using oligo-dT attached magnetic beads and 2 rounds of purification. During the second elution of the polyA RNA, the RNA is fragmented and primed for cDNA synthesis.

Consumables

- Total RNA samples (0.1–1 µg per sample)
- BBB (Bead Binding Buffer)
- BWB (Bead Washing Buffer)
- ELB (Elution Buffer)
- FPF (Fragment, Prime, Finish Mix)
- RPB (RNA Purification Beads)
- RSB (Resuspension Buffer)
- Barcode labels
  - RBP (RNA Bead Plate)
  - [HS] RFP (RNA Fragmentation Plate)
- Choose from the following containers:
  - [HS] 96-well Hard-Shell 0.3 ml PCR plate (1) and 96-well midi plate (1)
  - [LS] 96-well 0.3 ml PCR plate, semiskirted or skirtless
- Microseal 'B' adhesive seals

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
FPF	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
RSB	-25°C to -15°C	Thaw at room temperature. Store at 2°C to 8°C after the initial thaw.
BBB	2°C to 8°C	Thaw at room temperature. Return to storage after use.
BWB	2°C to 8°C	Thaw at room temperature. Return to storage after use.
ELB	2°C to 8°C	Thaw at room temperature. Return to storage after use.
RPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Save the following Elution 2-Frag-Prime program on the thermal cycler.
- Choose the preheat lid option and set to 100°C
- 94°C for 8 minutes
- Hold at 4°C

  For inserts larger than 120–200 bp with a median size of 150 bp, see *Alternate Fragmentation Protocols* on page 33.

3 [LS] Save the following mRNA Denaturation program on the thermal cycler.
- Choose the preheat lid option and set to 100°C
- 65°C for 5 minutes
- Hold at 4°C

4 [LS] Save the following mRNA Elution 1 program on the thermal cycler.
   - Choose the preheat lid option and set to 100°C
   - 80°C for 2 minutes
   - Hold at 25°C
5 [HS] Preheat the microheating system to 65°C.
6 Set the centrifuge to 15°C to 25°C.
7 [HS] Calibrate the microplate shaker to 1000 rpm using a stroboscope.
8 Apply barcode labels to plates as follows.
   - RBP [midi or PCR plate]
   - [HS] RFP [Hard-Shell PCR plate]

Procedure

Purify mRNA

1 Dilute the total RNA in nuclease-free ultrapure water to a final volume of 50 µl in each well of the RBP plate.
2 Vortex RPB until well-dispersed.
3 Add 50 µl RPB to each well, and then mix thoroughly as follows.
   - [HS] Shake at 1000 rpm for 1 minute.
   - [LS] Pipette up and down 6 times.
4 Incubate as follows.
   - [HS] Place on the 65°C microheating system with the lid closed for 5 minutes, and then place on ice for 1 minute.
   - [LS] Place on the thermal cycler and run the mRNA Denaturation program. Each well contains 100 µl.
5 Seal the RBP plate with a Microseal 'B' adhesive seal before running the mRNA denaturation program.
6 Place on the bench and incubate at room temperature for 5 minutes.
7 [HS] Preheat the microheating system to 80°C for subsequent incubation.
8 [LS] Centrifuge at 280 × g for 1 minute.
9 Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
10 Remove and discard all supernatant from each well.
11 Remove from the magnetic stand.
12 Add 200 µl BWB to each well, and then mix thoroughly as follows.
   - [HS] Shake at 1000 rpm for 1 minute.
   - [LS] Pipette up and down 6 times.
13 Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
14 Remove and discard all supernatant from each well.
15 Remove from the magnetic stand.
16 Add 50 µl ELB to each well, and then mix thoroughly as follows.
   - [HS] Shake at 1000 rpm for 1 minute.
   - [LS] Pipette up and down 6 times.

17 [LS] Centrifuge at 280 × g for 1 minute.

18 Incubate as follows.
- [HS] Place on the 80°C microheating system with the lid closed for 2 minutes, and then place on ice for 1 minute.
- [LS] Place on the thermal cycler and run the mRNA Elution 1 program. Each well contains 50 µl.

19 Place on the bench.

Fragment mRNA

1 Add 50 µl BBB to each well, and then mix thoroughly as follows.
- [HS] Shake at 1000 rpm for 1 minute.
- [LS] Pipette up and down 6 times.

2 Seal the RBP plate with a Microseal 'B' adhesive seal before running the mRNA denaturation program.

3 Incubate at room temperature for 5 minutes.

4 Place on a magnetic stand and wait until the liquid is clear (~5 minutes).

5 Remove and discard all supernatant from each well.

6 Remove from the magnetic stand.

7 Add 200 µl BWB to each well, and then mix thoroughly as follows.
- [HS] Shake at 1000 rpm for 1 minute.
- [LS] Pipette up and down 6 times.

8 Place on a magnetic stand and wait until the liquid is clear (~5 minutes).

9 Remove and discard all supernatant from each well.

10 Remove from the magnetic stand.

11 Add 19.5 µl FPF to each well, and then mix thoroughly as follows.
- [HS] Shake at 1000 rpm for 1 minute.
- [LS] Pipette up and down.

12 [LS] Centrifuge at 280 × g for 1 minute.

13 [HS] Transfer all to the corresponding well of the RFP plate.

14 Place on the thermal cycler and run the Elution 2 - Frag - Prime program. Each well contains 19.5 µl.

15 Centrifuge briefly.

Synthesize First Strand cDNA

This process reverse transcribes the cleaved RNA fragments primed with random hexamers into first strand cDNA. The addition of Actinomycin D to the FSA (First Strand Synthesis Act D Mix) prevents spurious DNA-dependent synthesis, while allowing RNA-dependent synthesis, and improving strand specificity.

Consumables

- FSA (First Strand Synthesis Act D Mix)
- CDP (cDNA Plate) barcode label
- SuperScript II Reverse Transcriptase or Protoscript II Reverse Transcriptase (Use part #18064-014 for 50 reactions. Make sure to have a quantity of two when using 96 samples)

- Choose from the following containers:
  - [HS] 96-well Hard-Shell 0.3 ml PCR plate
  - [LS] 96-well 0.3 ml PCR plate, semiskirted or skirtless
- Microseal 'B' adhesive seals

WARNING

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. See the safety data sheet (SDS) for environmental, health, and safety information. For more information, see *Technical Assistance* on page 35.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
FSA	-25°C to -15°C	Thaw at room temperature. Return to storage after use.

2 Save the following Synthesize 1st Strand program on the thermal cycler:
   - Choose the preheat 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

3 Apply the CDP barcode label to a Hard-Shell PCR or PCR plate.

Procedure

1 Place the RBP plate on the magnetic stand and wait until the liquid is clear (~5 minutes).

2 Transfer 17 µl supernatant to the corresponding well of the CDP plate.

3 Centrifuge FSA at 600 × g for 5 seconds.

4 Add 50 µl SuperScript II to one tube of FSA. Pipette to mix, and then centrifuge briefly.
Label the FSA tube to indicate that SuperScript II has been added.

NOTE

If you are not using the entire contents of FSA, add SuperScript II at a ratio of 1 µl SuperScript II to 9 µl FSA.

The mixture can be used for subsequent experiments. For more than 6 freeze-thaw cycles, prepare 10 µl aliquots and store at -25°C to -15°C.

5 Add 8 µl FSA and SuperScript II mixture to each well of the DFP plate, and then mix thoroughly as follows.
   - [HS] Shake at 1600 rpm for 20 seconds.
   - [LS] Pipette up and down.

6 Centrifuge at 280 × g for 1 minute.

7 Place on the preprogrammed thermal cycler and run the Synthesize 1st Strand program. Each well contains 25 µl.

Synthesize Second Strand cDNA

This process removes the RNA template, synthesizes a replacement strand, and incorporates dUTP in place of dTTP to generate ds cDNA. The incorporation of dUTP quenches the second strand during amplification. Magnetic beads separate the ds cDNA from the second strand reaction mix. The result is blunt-ended cDNA.

Consumables

- RSB (Resuspension Buffer)
- SMM (Second Strand Marking Master Mix)
- AMPure XP beads
- [Optional] CTE (End Repair Control)
- Barcode labels
  - ALP (Adapter Ligation Plate)
  - [HS] CCP (cDNA Clean Up Plate)
- Freshly prepared 80% ethanol (EtOH)
- Choose from the following containers:
  - [HS] 96-well midi plates (2)
  - [LS] 96-well 0.3 ml PCR plate, semiskirted or skirtless
- Microseal 'B' adhesive seals

About Reagents

- Using CTE is optional. Use equal volume of RSB as a substitute.
- Vortex AMPure XP beads before each use.
- Vortex AMPure XP beads frequently to make sure that beads are evenly distributed.
- Aspirate and dispense AMPure XP beads slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
CTE	-25°C to -15°C	Thaw at room temperature, and then place on ice.
SMM	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
AMPure XP beads	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Choose the thermal cycler preheat lid option and set the lid to 30°C

3 Preheat the thermal cycler to 16°C.

4 Apply barcode labels to plates as follows.
  - ALP [midi or PCR]
  - [HS] CCP [midi]

Procedure

Add SMM

1 Centrifuge CTE at 600 × g for 5 seconds.

2 Dilute CTE to 1:50 in RSB. For example, 2 µl CTE + 98 µl RSB.

3 Add 5 µl diluted CTE to each well. Discard diluted CTE after use.

4 Centrifuge SMM at 600 × g for 5 seconds.

5 Add 20 µl SMM to each well, and then mix thoroughly as follows.
- [HS] Shake at 1600 rpm for 20 seconds.
- [LS] Pipette up and down 6 times.

6 Centrifuge at 280 × g for 1 minute.

7 Place on the preprogrammed thermal cycler and incubate at 16°C for 1 hour. Each well contains 50 µl.

8 Place on the bench and let stand to bring to room temperature.

Purify cDNA

1 [HS] Add AMPure XP beads as follows.

a Add 90 µl AMPure XP beads to the CCP plate.
b Transfer all from the CDP plate to the corresponding well of the CCP plate.

2 [LS] Add 90 µl AMPure XP beads to each well of the CDP plate.

3 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down 10 times.

4 Incubate at room temperature for 15 minutes.

5 Centrifuge at 280 × g for 1 minute.

6 Place on a magnetic stand and wait until the liquid is clear (~5 minutes).

7 Remove and discard 135 µl supernatant from each well.

8 Wash two times as follows.

a Add 200 µl fresh 80% EtOH to each well.
b Incubate on the magnetic stand for 30 seconds.
c Remove and discard all supernatant from each well.

9 Use a 20 µl pipette to remove residual EtOH from each well.

10 Air-dry on the magnetic stand for 15 minutes. Do not over dry beads.

11 Remove from the magnetic stand.

12 Add 17.5 µl RSB to each well, and then mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down 10 times.

13 Incubate at room temperature for 2 minutes.

14 Centrifuge at 280 × g for 1 minute.

15 Place on a magnetic stand and wait until the liquid is clear (~5 minutes).

16 Transfer 15 µl supernatant to the corresponding well of the ALP plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Adenylate 3′ Ends

One adenine (A) nucleotide is added to the 3′ ends of the blunt fragments to prevent them from ligating to each other during adapter ligation reaction. One corresponding thymine (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

- ATL (A-Tailing Mix)
- RSB (Resuspension Buffer)
- [Optional] CTA (A-Tailing Control)
- Microseal 'B' adhesive seals

About Reagents

- Using CTA is optional. Use equal volume of RSB as a substitute.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
ATL	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
CTA	-25°C to -15°C	Thaw at room temperature, and then place on ice.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 [HS] Preheat two microheating systems, one to 37°C and the other to 70°C.

3 [LS] Save the following ATAIL70 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 37°C for 30 minutes
- 70°C for 5 minutes
- Hold at 4°C

Procedure

1 Centrifuge CTA at 600 × g for 5 seconds.

2 Dilute CTA to 1:100 in RSB. For example, 1 µl CTA + 99 µl RSB.

3 Add 2.5 µl diluted CTA to each well. Discard diluted CTA after use.

4 Centrifuge ATL at 600 × g for 5 seconds.

5 Add 12.5 µl ATL to each well, and then mix thoroughly as follows.
   - [HS] Shake at 1800 rpm for 2 minutes.
   - [LS] Pipette up and down 10 times.

6 Seal the ALP plate with a Mircoseal 'B' adhesive seal.

7 Centrifuge at 280 × g for 1 minute.

8 [HS] Incubate as follows.

   a Place on the 37°C microheating system with the lid closed for 30 minutes.
   b Move to the 70°C microheating system with the lid closed for 5 minutes.
   c Place on ice for 1 minute.

9 [LS] Incubate as follows.

   a Place on the thermal cycler and run the ATAIL70 program. Each well contains 30 µl.
   b Centrifuge at 280 × g for 1 minute.

Ligate Adapters

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

Index adapters must be ordered separately from the Library Prep components. For information on compatible index adapters, see *Supporting Information* on page 26.

Consumables

- LIG (Ligation Mix)
- RNA Adapters (tubes or index adapter plate)
- RSB (Resuspension Buffer)
- AMPure XP beads
- STL (Stop Ligation Buffer)
- [Optional] CTL (Ligation Control)
- Barcode labels
  - CAP (Clean Up ALP Plate)
  - PCR (Polymerase Chain Reaction Plate)
  - [HS workflow] RAP (Index Adapter Plate)
- Freshly prepared 80% ethanol (EtOH)
- Choose from the following containers:
  - [HS] 96-well midi plate and 96-well Hard-Shell 0.3 ml PCR plate
  - [LS] 96-well 0.3 ml PCR plates, semiskirted or skirtless (2)
- Microseal 'B' adhesive seals

About Reagents

- Using CTL is optional. Use an equal volume of RSB as a substitute.
- Do not remove LIG from storage until instructed to do so in the procedure.
- Return LIG to storage immediately after use.

- Vortex AMPure XP beads before each use.
- Vortex AMPure XP beads frequently to make sure that beads are evenly distributed.
- Aspirate and dispense AMPure XP beads slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
CTL	-25°C to -15°C	Thaw at room temperature, and then place on ice.
RNA Adapters	-25°C to -15°C	Thaw at room temperature for 10 minutes. Return to storage after use.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
AMPure XP beads	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
STL	-25°C to -15°C	Thaw at room temperature. Return to storage after use.

2 [HS] Preheat a microheating system to 30°C.

3 [LS] Save the following LIG program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 30°C for 10 minutes
- Hold at 4°C

4 Label plates as follows.
- Apply a CAP barcode label to a midi or PCR plate.
- Apply a PCR barcode label to a Hard-Shell PCR or PCR plate.

Procedure

Add Index Adapters

1 [HS] Remove the tape seal from the appropriate Index Adapter Plate.

2 Centrifuge the RNA Adapters as follows.

Reagent	Speed	Duration
Adapter tubes	600 × g	5 seconds
Index Adapter Plate	280 × g	1 minute

3 [HS] Prepare the Index Adapter Plate as follows.

a Remove the plastic cover.
b Apply the Index Adapter Plate barcode label.

4 Centrifuge CTL at 600 × g for 5 seconds.

5 Dilute CTL 1:100 in RSB. For example, 1 µl CTL + 99 µl RSB. Discard the diluted CTL after use.

6 Remove LIG from -25°C to -15°C storage.

7 Add the following reagents in the order listed to each well.
- Diluted CTL (2.5 µl)
- LIG (2.5 µl)
- RNA adapters (2.5 µl)

8 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down 10 times.

9 Centrifuge at 280 × g for 1 minute.

10 [HS] Place on the 30°C microheating system with the lid closed for 10 minutes, and then place on ice.

11 [LS] Place on the thermal cycler and run the LIG program. Each well contains 37.5 µl.

12 Centrifuge STL at 600 × g for 5 seconds.

13 Add 5 µl STL to each well, and then mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

14 Centrifuge at 280 × g for 1 minute.

Clean Up Ligated Fragments

1 Perform steps 2 through 17 using the **Round 1** volumes.

2 Add AMPure XP beads to each well.

	Round 1	Round 2
AMPure XP beads	42 µl	50 µl

3 Mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

4 Incubate at room temperature for 15 minutes.

5 Centrifuge at 280 × g for 1 minute.

6 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

7 Remove and discard all supernatant from each well.

8 Wash two times as follows.

a Add 200 µl fresh 80% EtOH to each well.
b Incubate on the magnetic stand for 30 seconds.
c Remove and discard all supernatant from each well.

9 Use a 20 µl pipette to remove residual EtOH from each well.

10 Air-dry on the magnetic stand for 15 minutes.

11 Remove from the magnetic stand.

12 Add RSB to each well.

	Round 1	Round 2
RSB	52.5 µl	22.5 µl

13 Mix thoroughly as follows.
   - [HS] Shake at 1800 rpm for 2 minutes.
   - [LS] Pipette up and down.

14 Incubate at room temperature for 2 minutes.

15 Centrifuge at 280 × g for 1 minute.

16 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

17 Transfer 50 µl supernatant to the corresponding well of the CAP plate.

18 Repeat steps 2 through 17 with the new plate using the **Round 2** volumes.

19 Transfer 20 µl supernatant to the corresponding well of the PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Enrich DNA Fragments

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

NOTE

Fragments with no adapters cannot hybridize to surface-bound primers in the flow cell. Fragments with an adapter on 1 end can hybridize to surface bound primers, but cannot form clusters.

Consumables

- PMM (PCR Master Mix)
- PPC (PCR Primer Cocktail)
- RSB (Resuspension Buffer)
- AMPure XP beads
- TSP1 (Target Sample Plate) barcode label
- Freshly prepared 80% ethanol (EtOH)
- Choose from the following containers:
  - [HS] 96-well Hard-Shell 0.3 ml PCR plate
  - [LS] 96-well 0.3 ml PCR plate, semiskirted or skirtless
- Microseal 'A' film
- Microseal 'B' adhesive seals

NOTE

Use Microseal 'A' when sealing the plate before placing it on the thermal cycler. Use Microseal 'B' for other steps that require a sealed plate.

About Reagents

- Vortex AMPure XP beads before each use.

- Vortex AMPure XP beads frequently to make sure that beads are evenly distributed.
- Aspirate and dispense AMPure XP beads slowly due to the viscosity of the solution.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
PPC	-25°C to -15°C	Thaw at room temperature. Invert to mix, then centrifuge at 600 × g for 1 minute. Do not vortex. Return to storage after use.
PMM	-25°C to -15°C	Thaw on ice. Invert to mix, then centrifuge at 600 × g for 1 minute. Do not vortex. Return to storage after use.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
AMPure XP beads	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Save the following mRNA PCR program on the thermal cycler:
   - Choose the preheat 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

3 Apply the TSP1 barcode label to a Hard-Shell PCR or PCR plate.

Procedure

Amplify DNA Fragments

1 Place PCR plate on ice and add 5 µl PPC to each well.

2 Add 25 µl PMM to each well, and then mix thoroughly as follows.
   - [HS] Shake at 1600 rpm for 20 seconds.
   - [LS] Pipette up and down 10 times.

3 Centrifuge at 280 × g for 1 minute.

4 Place on the preprogrammed thermal cycler and run the mRNA PCR program. Each well contains 50 µl.

Clean Up Amplified DNA

1 Centrifuge at 280 × g for 1 minute.

2 Add AMPure XP beads to each well. The volume depends on the type of adapter used.

Adapter Type	Volume AMPure XP beads
Adapter tubes	50 µl
Index Adapter Plate	47.5 µl

3 Mix thoroughly, as follows.
   - [HS] Shake at 1800 rpm for 2 minutes.
   - [LS] Pipette up and down 10 times.

4 Incubate at room temperature for 15 minutes.

5 Centrifuge at 280 × g for 1 minute.

6 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

7 Remove and discard all supernatant from each well.

8 Wash two times as follows.

   a Add 200 µl fresh 80% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

9 Use a 20 µl pipette to remove residual EtOH from each well.

10 Air-dry on the magnetic stand for 15 minutes.

11 Remove from the magnetic stand.

12 Add 32.5 µl RSB to each well, and then mix thoroughly as follows.
   - [HS] Shake at 1800 rpm for 2 minutes.
   - [LS] Pipette up and down 10 times.

13 Incubate at room temperature for 2 minutes.

14 Centrifuge at 280 × g for 1 minute.

15 Place on a magnetic stand and wait until the liquid is clear (2–5 minutes).

16 Transfer 30 µl supernatant to the corresponding well of the TSP1 plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Check Libraries

Quantify Libraries

To achieve the highest-quality data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of the flow cell. Optimizing cluster densities requires accurate quantification of DNA libraries.

1 Quantify the libraries using qPCR according to the *IluminaSequencing Library qPCR Quantification Guide (document # 11322363)*.

Check Library Quality

1 If using a Standard Sensitivity NGS Fragment Analysis Kit on an Advanced Analytical Fragment Analyzer:

   a Dilute the DNA library 1:1 with RSB.
   b Run 1 µl diluted DNA library.

2 If using a DNA 1000 chip on an Agilent Technologies 2100 Bioanalyzer, run 1 µl undiluted DNA library.

3 Check the size and purity of the sample. Expect the final product to be a band at ~260 bp.

Figure 12 Example Library Size Distribution

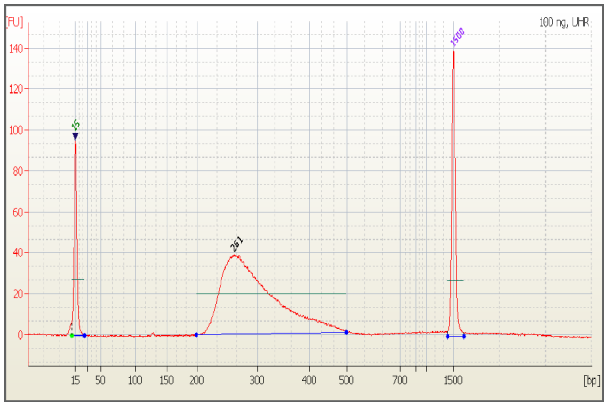
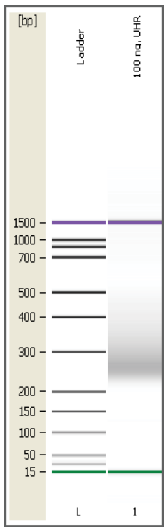


Figure 13 TruSeq Stranded mRNA Library Prep 260 bp PCR Product



Normalize and Pool Libraries

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

NOTE

For best practice, perform normalization and pooling directly prior to sequencing. To minimize index hopping, do not store libraries in the pooled form. For more information, see *Minimize index hopping in multiplexed runs* on the Illumina website.

Consumables

- Barcode labels
  - DCT (Diluted Cluster Template)
  - PDP (Pooled DCT Plate) (for pooling only)

- Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20
- Choose from the following containers:
  - [HS] 96-well midi plate
  - [HS] 96-well Hard-Shell 0.3 ml PCR plate (for pooling)
  - [LS] 96-well midi plates (2) (second plate for pooling > 40 samples)
  - [LS] 96-well 0.3 ml PCR plate, semiskirted or skirtless (for pooling ≤ 40 samples)
- Microseal 'B' adhesive seals

Preparation

1 Apply barcode labels to plates as follows.
   - DCT [midi plate]
   - [For pooling only] PDP [Hard-Shell PCR or midi (> 40 samples) or PCR (≤ 40 samples) plate]

Procedure

Normalize Libraries

1 Transfer 10 µl library to the corresponding well of the DCT plate.

2 Normalize the library concentration with Tris-HCl 10 mM, pH 8.5 with 0.1% Tween 20 to 10 nM, and then mix thoroughly as follows.
   - [HS] Shake at 1000 rpm for 2 minutes.
   - [LS] Pipette up and down 10 times.

NOTE

Depending on the yield quantification data of each library, the final volume of each well can vary from 10–400 µl.

3 Centrifuge at 280 × g for 1 minute.

4 Do the following,
   - To pool libraries, proceed to the next step in the workflow.
   - Libraries that are not pooled, must be diluted and denatured before proceeding to cluster generation. For more information, see the Dilute and Denature guide for your Illumina platform.

Pool Libraries

The pooling procedure depends on the number of libraries being pooled.

Pool 2–24 Libraries

1 Transfer 10 µl of each normalized library to a single well of the PDP plate.

2 Mix thoroughly as follows.
   - [HS] Shake at 1800 rpm for 2 minutes.
   - [LS] Pipette up and down 10 times.

3 [HS] Centrifuge at 280 × g for 1 minute.

4 Proceed to cluster generation. For more information, see the system guide for your Illumina sequencing platform.

Pool 25–96 Libraries

1 Transfer 5 µl of each column of normalized library to column 1 of the PDP plate, and then mix thoroughly as follows.
   - [HS] Shake at 1800 rpm for 2 minutes.
   - [LS] Pipette up and down 10 times.

2 Centrifuge at 280 × g for 1 minute.

3 Transfer the contents of each well of column 1 to well A2.

4 Mix thoroughly as follows.
   - [HS] Shake at 1800 rpm for 2 minutes.
   - [LS] Pipette up and down 10 times.

5 Centrifuge at 280 × g for 1 minute.

6 Proceed to cluster generation. For more information, see the system guide for your Illumina sequencing platform.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Supporting Information

Introduction

The protocol described in this guide assumes that you have reviewed the contents of this section, confirmed workflow contents, and obtained all required consumables and equipment.

Product Contents

Make sure that you have all the reagents identified in this section before starting the protocol.

The following library prep and index adapter components are available to order through Illumina to support the TruSeq Stranded mRNA library prep workflow.

From Illumina, order one catalog number for the library prep component and one catalog number for the index adapter component depending on the number of samples for your experiment.

Library Prep Component	Catalog #
TruSeq Stranded mRNA Library Prep (48 Samples)	20020594
TruSeq Stranded mRNA Library Prep (96 Samples)	20020595

Index Adapter Component	Catalog #
IDT for Illumina-TruSeq RNA UD Indexes (24 indexes, 96 samples)	20020591
IDT for Illumina-TruSeq RNA UD Indexes (96 indexes, 96 samples)	20022371
TruSeq RNA Combinatorial Dual Indexes (96 indexes, 96 samples)	20019792
TruSeq RNA Single Indexes (12 indexes, 24 samples) Set A	20020492
TruSeq RNA Single Indexes (12 indexes, 24 samples) Set B	20020493

TruSeq Stranded mRNA Library Prep (48 Samples)

This workflow uses the components described in the sections that follow.

Library Prep Box 1, Store as specified

Quantity	Reagent	Description	Storage Temperature
1	RPB	RNA Purification Beads	2°C to 8°C
1	DTE	CTE Dilution Tube	Room Temperature
1	DTA	CTA Dilution Tube	Room Temperature
1	DTL	CTL Dilution Tube	Room Temperature

Library Prep Box 2, Store as specified

This box also contains plate barcode labels.

Quantity	Reagent	Description	Storage Temperature
1	BBB	Bead Binding Buffer	2°C to 8°C
1	BWB	Bead Washing Buffer	2°C to 8°C
1	ELB	Elution Buffer	2°C to 8°C
1	FPF	Fragment, Prime, Finish Mix	-25°C to -15°C

Core Library Prep Box 1, Store at -25°C to -15°C

Quantity	Reagent	Description
1	ATL	A-Tailing Mix
1	CTA	A-Tailing Control
1	CTE	End Repair Control
1	CTL	Ligation Control
1	LIG	Ligation Mix
1	RSB	Resuspension Buffer
1	STL	Stop Ligation Buffer

Core Library Prep Box 2, Store at -25°C to -15°C

Quantity	Reagent	Description
1	PMM	PCR Master Mix
1	PPC	PCR Primer Cocktail
1	FSA	First Strand Synthesis Act D Mix
1	SMM	Second Strand Marking Master Mix

TruSeq Stranded mRNA Library Prep (96 Samples)

This workflow uses the components described in the sections that follow. A quantity of two of each box is included for the 96 sample workflow.

Library Prep Box 1, Store as specified

Quantity	Reagent	Description	Storage Temperature
2	RPB	RNA Purification Beads	2°C to 8°C
1	DTL	CTL Dilution Tube	Room Temperature
1	DTE	CTE Dilution Tube	Room Temperature
1	DTA	CTA Dilution Tube	Room Temperature

Library Prep Box 2, Store as specified

This box also contains plate barcode labels.

Quantity	Reagent	Description	Storage Temperature
2	BBB	Bead Binding Buffer	2°C to 8°C
2	ELB	Elution Buffer	2°C to 8°C
2	BWB	Bead Washing Buffer	2°C to 8°C
2	FPF	Fragment, Prime, Finish Mix	-25°C to -15°C

Core Library Prep Box 1, Store at -25°C to -15°C

Quantity	Reagent	Description
1	ATL	A-Tailing Mix
1	CTA	A-Tailing Control
1	CTE	End Repair Control
1	CTL	Ligation Control
1	LIG	Ligation Mix
1	RSB	Resuspension Buffer
1	STL	Stop Ligation Buffer

Core Library Prep Box 2, Store at -25°C to -15°C

Quantity	Reagent	Description
2	PMM	PCR Master Mix
2	PPC	PCR Primer Cocktail
2	FSA	First Strand Synthesis Act D Mix
2	SMM	Second Strand Marking Master Mix

Inline Control DNA

The use of the included In-line Control DNA provided with this kit is optional and requires a custom analysis pipeline. If analysis is not available, Illumina recommends omitting them from the prep.

CTE (End Repair Control), CTA (A-Tailing Control), and CTL (Ligation Control) contain DNA fragments used as controls for the enzymatic activities of the SMM (Second Strand Marking Master Mix), ATL (A-Tailing Mix), and LIG (Ligation Mix). Each inline control contains dsDNA fragments designed to report the success or failure of a specific enzymatic activity.

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

Table 2 Inline Control Functions

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

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

Inline controls can be used for various library insert sizes. Each is provided in ladders ranging from approximately 150–850 bp in 100 bp increments. Each control molecule has a unique DNA sequence, which indicates both its function and size.

Inline controls are used for troubleshooting and to identify the specific mode of failure. Using controls is optional. You can replace inline controls with an equal volume of RSB.

Consumables and Equipment

Some items required depend on the workflow performed (HS or LS) and these items are specified in separate tables.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Consumables

Consumable	Supplier
1.5 ml RNase/DNase-free nonsticky tubes	Thermo Fisher Scientific, 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)	Thermo Fisher Scientific, part # AB-0859
Agencourt AMPure XP 60 ml kit	Beckman Coulter Genomics, part # A63881
Agilent DNA 1000 Kit	Agilent Technologies, part # 5067-1504
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023

Consumable	Supplier
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
Nuclease-free ultrapure water	General lab supplier
RNaseZap (to decontaminate surfaces)	General lab supplier
RNase/DNase-free 8-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
SuperScript II Reverse Transcriptase (1 per 48 reactions)	Thermo Fisher Scientific, part # 18064-014
Tris-HCl 10 mM, pH8.5	General lab supplier
Tween 20	Sigma, part # P7949
[Optional - to aliquot reagents] 96-well 2 ml deep well plates	Thomson Instrument Company, part # 951652
[Optional - to determine input RNA integrity] Certified low range ultra agarose	Bio-Rad, part # 161-3107
[Optional - positive control] Human UHR total RNA	Agilent Technologies, part # 740000
[Optional - for starting material quality assessment] One of the following: • Standard Sensitivity RNA Analysis Kit (20nt Lower Marker) • Agilent RNA 6000 Nano Kit	• Advanced Analytical Technologies, part # DNF-489 • Agilent Technologies, part # 5067-1511

Consumables for HS Workflow

Consumable	Supplier
96-well Hard-Shell 0.3 ml PCR plate	Bio-Rad, part # HSP-9601
Microseal 'A' film	Bio-Rad, part # MSA-5001

Consumables for LS Workflow

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

Equipment

Equipment	Supplier/Description
96-well thermal cycler (with programmable heated lid)	General lab supplier
One of the following: • Fragment Analyzer Automated CE System • 2100 Bioanalyzer Desktop System	• Advanced Analytical Technologies, part # FSv2-CE2 or FSv2-CE10 • Agilent Technologies, part # G2940CA
Magnetic stand-96	Thermo Fisher Scientific, part # AM10027
Microplate centrifuge	General lab supplier
Vortexer	General lab supplier

Equipment for HS Workflow

Consumable	Supplier
High-Speed Microplate Shaker	VWR, catalog # • 13500-890 (110 V/120 V) or • 14216-214 (230 V)
Midi plate insert for heating system Note: Two inserts are recommended to support successive heating procedures.	Illumina, catalog # BD-60-601
Stroboscope	General lab supplier
One of the following: Note: Two systems are recommended to support successive heating procedures. • SciGene TruTemp Heating System • Hybex Microsample Incubator	 • Illumina, catalog # SC-60-503 (115 V) or SC-60-504 (220 V) • SciGene, catalog # 1057-30-0 (115 V) or 1057-30-2 (230 V)

Index Adapter Sequences

For information on index adapter sequences, see *Illumina Adapter Sequences (document # 1000000002694)* which provides information regarding the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.

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
CDP	cDNA Plate
CTA	A-Tailing Control
CTE	End Repair Control
CTL	Ligation Control
DCT	Diluted Cluster Template
ELB	Elution Buffer
FPF	Fragment, Prime, Finish Mix
FSA	First Strand Synthesis Act D Mix
HS	High Sample
IEM	Illumina Experiment Manager
LIG	Ligation Mix

Acronym	Definition
LRM	Local Run Manager
LS	Low Sample
PCR	Polymerase Chain Reaction
PDP	Pooled Dilution Plate
PMM	PCR Master Mix
PPC	PCR Primer Cocktail
RBP	RNA Bead Plate
RFP	RNA Fragmentation Plate
RPB	RNA Purification Beads
RSB	Resuspension Buffer
SSM	Second Strand Master Mix
STL	Stop Ligation Buffer
TSP	Target Sample Plate

Alternate Fragmentation Protocols

Introduction

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

Some studies require larger insert sizes, such as splice variant analysis studies. Modified the fragmentation times allow for varying the insert size of your library.

Modify RNA Fragmentation Time

To modify the fragmentation of the RNA to allow for longer RNA fragments, the time of fragmentation can be shortened during *Purify and Fragment mRNA* on page 9. Modify the thermal cycler Elution 2 - Frag - Prime program: 94°C for X minutes followed by a 4°C hold for the thermal cycler. Determine X based on the length of the desired RNA. See Table 3 for a range of suggested times and sizes.

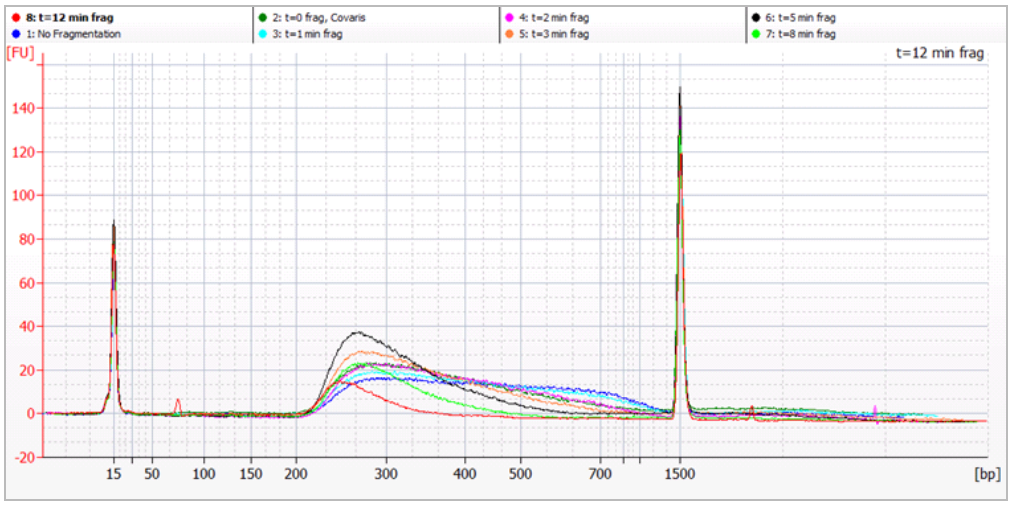
Table 3 Library Insert Fragmentation Time

Time at 94 °C (minutes)	Range of Insert Length[a] (bp)	Median Insert Length[a] (bp)	Average Final Library Size (Bioanalyzer bp)
0[b]	130–350	200	467
1	130–310	190	439
2	130–290	185	410
3	125–250	165	366
4	120–225	160	326
8	120–210	155	309
12	115–180	140	272
Covaris[c]	130–280	180	385

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

b. Skip the Incubate RFP procedure (fragmentation) for samples requiring 0 minutes fragmentation time. Instead, place the sealed plate on the preheated thermal cycler. Close the lid and incubate the plate at 80°C for 2 minutes to elute full length RNA from the RNA purification beads. Then, immediately place the plate on the magnetic stand and proceed to the Synthesize First Strand cDNA process.

Figure 14 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.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

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

Illumina Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
Australia	+1.800.775.688	
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Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

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