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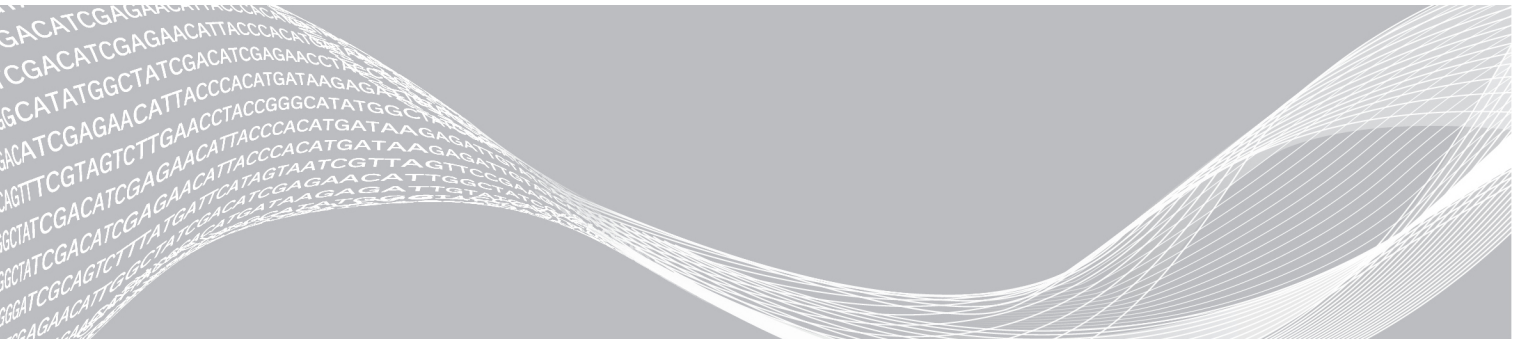
# TruSeq Stranded Total RNA

## Reference Guide



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# Chapter 1 Overview



## Introduction

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

- The following support human, mouse, and rat organisms:
  - TruSeq Stranded Total RNA Library Prep workflow with Ribo-Zero™ Globin
  - TruSeq Stranded Total RNA Library Prep workflow with Ribo-Zero Gold
  - TruSeq Stranded Total RNA Library Prep workflow with Ribo-Zero Human/Mouse/Rat
- TruSeq Stranded Total RNA Library Prep workflow with Ribo-Zero Plant supports plant species

This library prep protocol offers:

- Strand information on RNA transcript
- Library capture of both coding RNA, as well as multiple forms of noncoding RNA
- Degraded RNA can be used with minor adjustments to fragmentation procedures
- 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 27.
  - 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.

## Process

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

### Deplete and Fragment RNA

Ribosomal RNA (rRNA) is removed using biotinylated, target-specific oligos combined with Ribo-Zero rRNA removal beads.

- Ribo-Zero Globin depletes globin-encoding mRNA in addition to the rRNA species targeted with Ribo-Zero Gold.
- Ribo-Zero Gold depletes samples of both cytoplasmic and mitochondrial rRNA.
- Ribo-Zero Human/Mouse/Rat depletes samples of cytoplasmic rRNA.
- Ribo-Zero Plant targets cytoplasmic and chloroplast rRNA.

Following purification, the RNA is fragmented into small pieces using divalent cations under elevated temperature.

**Figure 1** Ribo-Zero Depleting and Fragmenting RNA


## Synthesize First Strand cDNA

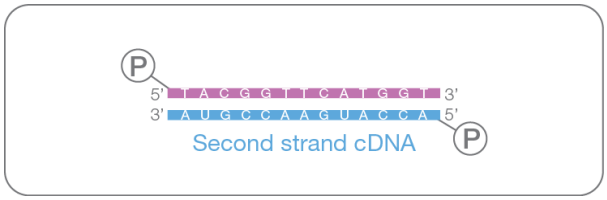
Cleaved RNA fragments are copied into first strand cDNA using reverse transcriptase and random primers. Adding Actinomycin D to FSA (First Stand Synthesis Act D mix) prevents spurious DNA-dependent synthesis, while allowing RNA-dependent synthesis, improving strand specificity.

**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.

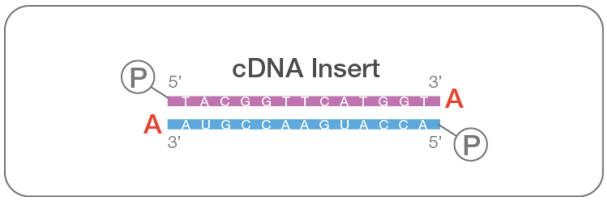
**Figure 3** Synthesizing Second Strand cDNA



## 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.

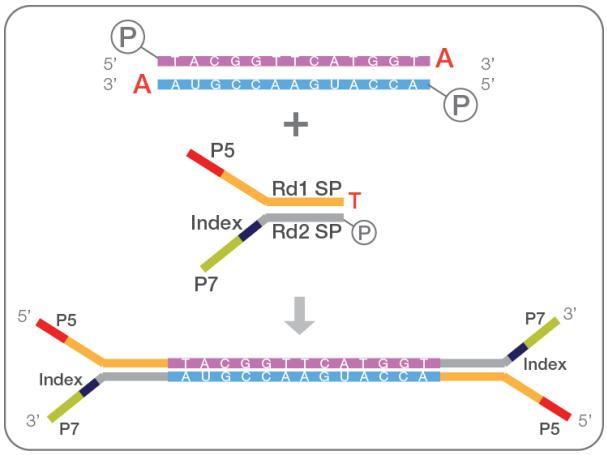
**Figure 4** Adenylating 3' Ends




## 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.

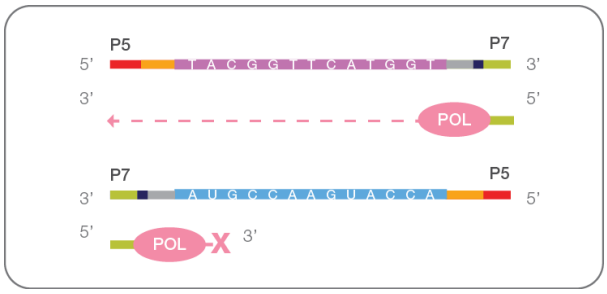
**Figure 5** Ligating Adapters



## 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.

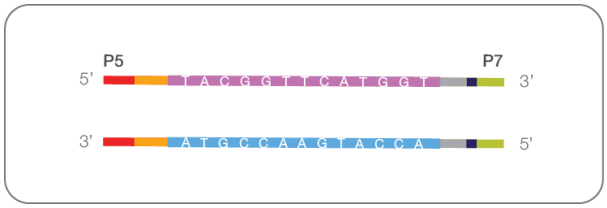
**Figure 6** Enriching DNA Fragments




## 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.

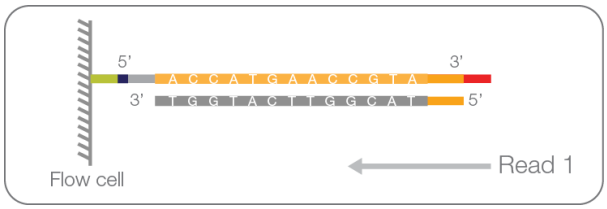
**Figure 7** LS Final Library




## Cluster Generation and Read 1 Sequencing

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

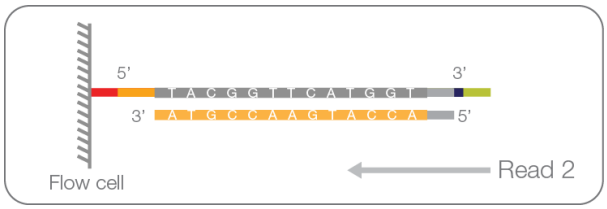
**Figure 8** Cluster Generation and Read 1 Sequencing



## 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



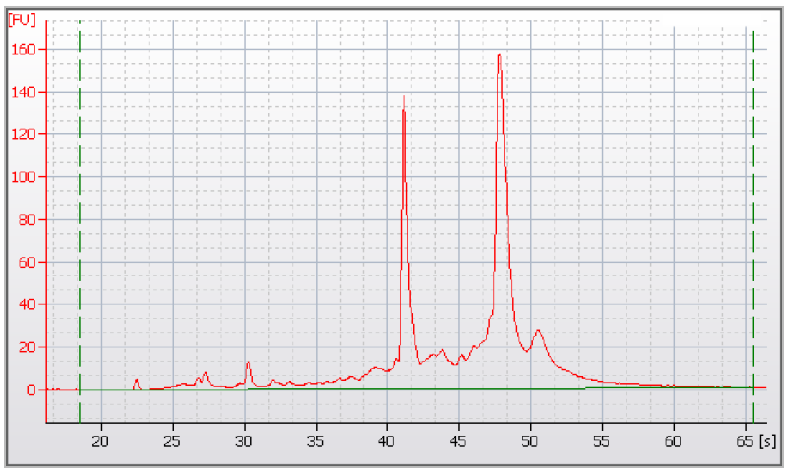

# RNA Input Recommendations

## Total RNA Input

- The protocol is optimized for 0.1–1 µg of total RNA.
  - Lower amounts can result in inefficient ligation and low yield.
  - Quantify RNA with a fluorimetric method.
- The protocol has been tested using 0.1–1 µg of the following total RNA input:
  - Ribo-Zero Globin – Total RNA isolated from human blood
  - Ribo-Zero Gold – UHR
  - Ribo-Zero (Human/Mouse/Rat) – Universal Human Reference (UHR) RNA
  - Ribo-Zero Plant – Arabidopsis Total RNA
- The protocol has been tested using 100 ng FFPE RNA.
  - Use of degraded or FFPE RNA can require further optimization of the initial input amount, depending on the quality of the sample.
  - Degraded or FFPE RNA is shorter than full length RNA. If the same fragmentation conditions for degraded RNA are used, libraries are shortened and can result in low yield or failure of the protocol.
    - RNA that has DNA contamination results in an underestimation of the amount of RNA used.
    - If starting with degraded RNA, adjust the fragmentation time to avoid over fragmentation of the RNA samples. For more information, see *Alternate Fragmentation Protocols* on page 36.
- Include a DNase step with the RNA isolation method to ensure purity and accurate quantification of the sample.

- Determine the quality of the RNA starting material. The fragmentation conditions are optimized for high-quality RNA.
  - Use the Agilent RNA 6000 Nano Kit or Advanced Analytical Standard Sensitivity RNA Analysis Kit to determine the quality of your starting material.
- The following figure shows a UHR starting RNA Bioanalyzer trace.

**Figure 10** Starting RNA Bioanalyzer Trace




## rRNA-Depleted Total RNA Input

You can use 10–100 ng of previously rRNA-depleted total RNA in a final volume of 8.5 µl of nuclease-free water or ELB (Elution Buffer) as starting material. Diluted samples require concentration, which can result in sample loss. Quantify RNA with a flurometric method.

1 Concentrate samples by ethanol precipitation or use a QIAGEN MinElute column.
   - If ethanol precipitation is used, resuspend the pellet in 8.5 µl ELB.
   - If a QIAGEN MinElute column is used, elute the RNA with 10 µl ELB and recover 8.5 µl.
2 Proceed to *Clean Up RNA* on page 13 step 16.

## 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<br>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 Total RNA Checklist (document # 10000000xxxxx)* | Provides a checklist of the protocol steps. The checklist is intended for experienced users. |

| Resource | Description |
|---|---|
| *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 #10000002701)* | 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 Total RNA 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<br>96-well midi plate | 96-well 0.3 ml PCR plate<br>96-well midi plate |
| Incubation Equipment | 96-well thermal cycler<br>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 Total RNA 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 27.

## 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***.
- 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. 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 Total RNA 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.

## Deplete and Fragment RNA

This process depletes rRNA from total RNA. After the rRNA is depleted, the remaining RNA is purified, fragmented, and primed for cDNA synthesis.

Successful depletion can be assessed by evaluating the rRNA peaks in the input sample before and after depletion on a Bioanalyzer or equivalent method. Successful depletion is indicated by the disappearance of the ribosomal rRNA peaks on the trace.

## Consumables

- ELB (Elution Buffer)
- EPH (Elute, Prime, Fragment High Mix)
- One of the following, depending on the workflow:
  - GRM (Globin Removal Mix)
  - RRM (rRNA Removal Mix)
  - RRM G (rRNA Removal Mix - Gold)
  - RRM P (rRNA Removal Mix - Plant)
- RBB (rRNA Binding Buffer)
- RRB (rRNA Removal Beads)
- RSB (Resuspension Buffer)
- Barcode labels
  - BRP (Bind rRNA Plate)
  - DFP (Depleted RNA Fragmentation Plate)
  - RCP (RNA Clean Up Plate)
  - RRP (rRNA Removal Plate)
- Freshly prepared 70% ethanol (EtOH)
- RNAClean XP Beads
- Ultrapure Water
- Choose from the following containers:
  - [HS] 96-well midi plates (2) and 96-well Hard-Shell 0.3 ml PCR plates (2)
  - [LS] 96-well 0.3 ml PCR plates, semiskirted or skirtless (4)
- Microseal 'B' adhesive seals

## About Reagents

- Allow RRB and RNAClean XP Beads to fully pellet against the magnetic stand for 1 minute and 5 minutes, respectively. Immediately remove the supernatant from the beads while the beads are still pelleted against the magnetic stand. Do not allow the RRB pellets to dry.
- After adding RRB:
  - Pipette up and down quickly to ensure thorough mixing. Insufficient mixing leads to lower levels of rRNA depletion.
  - Pipette with the tips at the bottom of the well to prevent foaming. Excess foam leads to sample loss because foam does not transfer from the plate efficiently.
- RNAClean XP Bead wash steps use 70% ethanol.

## Preparation

1 Prepare the following consumables.

| Item | Storage | Instructions |
|---|---|---|
| EPH | -25°C to -15°C | Thaw at room temperature.<br>Return to storage after use. |
| One of the following:<br>GRM, RRM, RRM G, RRM P | -25°C to -15°C | Thaw at room temperature.<br>Return to storage after use. |
| RBB | -25°C to -15°C | Thaw at room temperature.<br>Return to storage after use. |
| RSB | -25°C to -15°C | Thaw at room temperature.<br>Store at 2°C to 8°C after the initial thaw. |
| ELB | 2°C to 8°C | Let stand to bring to room temperature.<br>Return to storage after use. |
| RRB | 2°C to 8°C | Let stand to bring to room temperature.<br>Return to storage after use. |
| RNAClean XP Beads | 2°C to 8°C | Let stand for 30 minutes to bring to room temperature.<br>Return to storage after use. |

2 Save the following RNA Denaturation program on the thermal cycler.
- Choose the preheat lid option and set to 100°C
- 68°C for 5 minutes
- Hold at 4°C

3 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 or if starting with degraded total RNA, see *Alternate Fragmentation Protocols* on page 36.

4 Set the centrifuge to 15°C to 25°C.

5 [HS] Calibrate the microplate shaker to 1000 rpm using a stroboscope.

6 Apply barcode labels to plates as follows.
- BRP [Hard-Shell PCR or PCR plate]
- DFP [Hard-Shell PCR or PCR plate]
- RCP [midi or PCR plate]
- RRP [midi or PCR plate]

## Procedure

### Bind rRNA

1 Dilute the total RNA in nuclease-free ultrapure water to a final volume of 10 µl in each well of the BRP plate.

2 Add 5 µl RBB to each well.

3 Add 5 µl of the appropriate removal mix to each well, depending on the workflow used.
   - GRM
   - RRM
   - RRM G
   - RRM P

4 Mix thoroughly as follows.
   - [HS] Shake at 1600 rpm for 20 seconds.
   - [LS] Pipette up and down.

5 Centrifuge at 280 × g for 1 minute.

6 Place on the thermal cycler and run the RNA Denaturation program. Each well contains 20 µl of sample.

7 Place on the bench and incubate at room temperature for 1 minute.

## Remove rRNA

1 Vortex RRB until well-dispersed.

2 Add 35 µl RRB to each well of the RRP plate.

3 Transfer all (20 µl) from each well of the BRP plate to the corresponding well of the RRP plate, and then mix thoroughly as follows.
   - [HS] Shake at 1000 rpm for 1 minute.
   - [LS] Pipette up and down.

4 [LS] Incubate at room temperature for 1 minute.

5 Place on a magnetic stand for 1 minute.

6 Transfer all to the corresponding well of the RCP plate.

7 Place on a magnetic stand for 1 minute.

## Clean Up RNA

1 Vortex RNAClean XP Beads until well dispersed.

2 Add 99 µl RNAClean XP Beads to each well, and then mix thoroughly as follows.
   - [HS] Shake at 1800 rpm for 2 minutes.
   - [LS] Pipette up and down.

**NOTE**

If starting with degraded total RNA, add 193 µl RNAClean XP Beads to each well.

3 Incubate at room temperature for 15 minutes.

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

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

6 Wash as follows.

   a Add 200 µl freshly prepared 70% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

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

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

9 Remove from the magnetic stand.

10 Centrifuge ELB to 600 × g for 5 seconds.

11 Add 11 µl ELB to each well, and then mix thoroughly as follows.
- [HS] Shake at 1800 rpm for 2 minutes.
- [LS] Pipette up and down.

12 Incubate at room temperature for 2 minutes.

13 Centrifuge at 280 × g for 1 minute.

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

15 Transfer 8.5 µl supernatant to the corresponding well of the DFP plate.

16 Add 8.5 µl EPH to each well, and then mix thoroughly as follows.
- [HS] Shake at 1600 rpm for 20 seconds.
- [LS] Pipette up and down.

17 Place on the thermal cycler and run the Elution 2-Frag-Prime program. Each well contains 17 µl of sample.

18 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)
- 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)
- 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 41.

## Preparation

1 Prepare the following consumables.

| Item | Storage | Instructions |
|---|---|---|
| FSA | -25°C to -15°C | Thaw at room temperature.<br>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 Centrifuge FSA at 600 × g for 5 seconds.
2 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.

3 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.
4 Centrifuge at 280 × g for 1 minute.
5 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.<br>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 Add 5 µl RSB to each well.

5 Centrifuge SMM at 600 × g for 5 seconds.

6 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.

7 Centrifuge at 280 × g for 1 minute.

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

9 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 DFP plate to the corresponding well of the CCP plate.

2 [LS] Add 90 µl AMPure XP beads to each well of the DFP 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.<br>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 27.

### 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.<br>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.<br>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.<br>Invert to mix, then centrifuge at 600 × g for 1 minute. Do not vortex.<br>Return to storage after use. |
| PMM | -25°C to -15°C | Thaw on ice.<br>Invert to mix, then centrifuge at 600 × g for 1 minute. Do not vortex.<br>Return to storage after use. |
| RSB | 2°C to 8°C | Let stand for 30 minutes to bring to room temperature. |

| Item | Storage | Instructions |
|---|---|---|
| AMPure XP beads | 2°C to 8°C | Let stand for 30 minutes to bring to room temperature. |

2 Save the following 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 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 (preferred method) according to the Illumina *Sequencing Library qPCR Quantification Guide (document # 11322363)* or fluorometric method.

## 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.

# 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 of 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 Total RNA 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 Total RNA Library Prep Human/Mouse/Rat (48 Samples) | 20020596 |
| TruSeq Stranded Total RNA Library Prep Human/Mouse/Rat (96 Samples) | 20020597 |
| TruSeq Stranded Total RNA Library Prep Gold (48 Samples) | 20020598 |
| TruSeq Stranded Total RNA Library Prep Gold (96 Samples) | 20020599 |
| TruSeq Stranded Total RNA Library Prep Plant (48 Samples) | 20020610 |
| TruSeq Stranded Total RNA Library Prep Plant (96 Samples) | 20020611 |
| TruSeq Stranded Total RNA Library Prep Globin (48 Samples) | 20020612 |
| TruSeq Stranded Total RNA Library Prep Globin (96 Samples) | 20020613 |

| 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 Total RNA Library Prep (48 Samples)

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

## Library Prep Box 1, Store as specified

**Table 2 TruSeq Stranded Total RNA library preparation, 48 samples**

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 1 | DTE | CTE Dilution Tube | Room Temperature |
| 1 | DTA | CTA Dilution Tube | Room Temperature |
| 1 | DTL | CTL Dilution Tube | Room Temperature |
| 1 | RRB | rRNA Removal Beads | 2°C to 8°C |

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

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 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 | |

## Library Prep Ribo-Zero Box, Store as specified

You receive one of the following boxes, depending on the workflow. The box also contains plate barcode labels.

**Table 3 Ribo-Zero H/M/R**

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 1 | RBB | rRNA Binding Buffer | -25°C to -15°C |
| 1 | RRM | rRNA Removal Mix | -25°C to -15°C |
| 1 | ELB | Elution Buffer | 2°C to 8°C |
| 1 | EPH | Elute, Prime, Fragment High Mix | -25°C to -15°C |

**Table 4 Ribo-Zero Gold**

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 1 | RBB | rRNA Binding Buffer | -25°C to -15°C |
| 1 | RRM G | rRNA Removal Mix - Gold | -25°C to -15°C |
| 1 | ELB | Elution Buffer | 2°C to 8°C |
| 1 | EPH | Elute, Prime, Fragment High Mix | -25°C to -15°C |

**Table 5 Ribo-Zero Plant**

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 1 | RBB | rRNA Binding Buffer | -25°C to -15°C |
| 1 | RRM P | rRNA Removal Mix - Plant | -25°C to -15°C |

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 1 | ELB | Elution Buffer | 2°C to 8°C |
| 1 | EPH | Elute, Prime, Fragment High Mix | -25°C to -15°C |

**Table 6 Ribo-Zero Globin**

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 1 | RBB | rRNA Binding Buffer | -25°C to -15°C |
| 1 | GRM | Globin Removal Mix | -25°C to -15°C |
| 1 | ELB | Elution Buffer | 2°C to 8°C |
| 1 | EPH | Elute, Prime, Fragment High 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 Total RNA 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 | RRB | rRNA Removal 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 at -25°C to -15°C

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 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 | |

## Library Prep Ribo-Zero Box, Store as specified

You receive a quantity of two of one of the following, depending on the workflow. The box also contains plate barcode labels.

**Table 7 Library Preparation H/M/R (48 Samples)**

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 2 | RBB | rRNA Binding Buffer | -25°C to -15°C |
| 2 | RRM | rRNA Removal Mix | -25°C to -15°C |
| 2 | ELB | Elution Buffer | 2°C to 8°C |
| 2 | EPH | Elute, Prime, Fragment High Mix | -25°C to -15°C |

**Table 8 Library Preparation Gold (48 Samples)**

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 2 | RBB | rRNA Binding Buffer | -25°C to -15°C |
| 2 | RRM G | rRNA Removal Mix - Gold | -25°C to -15°C |
| 2 | ELB | Elution Buffer | 2°C to 8°C |
| 2 | EPH | Elute, Prime, Fragment High Mix | -25°C to -15°C |

**Table 9 Library Preparation Plant (48 Samples)**

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 2 | RBB | rRNA Binding Buffer | -25°C to -15°C |
| 2 | RRM P | rRNA Removal Mix - Plant | -25°C to -15°C |
| 2 | ELB | Elution Buffer | 2°C to 8°C |
| 2 | EPH | Elute, Prime, Fragment High Mix | -25°C to -15°C |

**Table 10 Library Preparation Globin (48 Samples)**

| Quantity | Reagent | Description | Storage Temperature |
|---|---|---|---|
| 2 | RBB | rRNA Binding Buffer | -25°C to -15°C |
| 2 | GRM | Globin Removal Mix | -25°C to -15°C |
| 2 | ELB | Elution Buffer | 2°C to 8°C |
| 2 | EPH | Elute, Prime, Fragment High 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 100 ng of starting material, the controls yield approximately 0.2% of clusters, although the yield can vary based on library yield.

Table 11 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 |
| Agencourt RNAClean XP 40 ml kit | Beckman Coulter Genomics, part # A63987 |
| Agilent DNA 1000 Kit | Agilent Technologies, part # 5067-1504 |

| Consumable | Supplier |
|---|---|
| Ethanol 200 proof (absolute) for molecular biology (500 ml) | Sigma-Aldrich, part # E7023 |
| 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 - for alternative fragmentation] One of the following:<br>• Agilent RNA 6000 Nano Kit<br>• Agilent RNA 6000 Pico Kit | Agilent Technologies<br>• part # 5067-1511<br>• part # 5067-1513 |
| [Optional - positive control] Human UHR total RNA | Agilent Technologies, part # 740000 |
| [Optional - for alternative fragmentation] MicroTube (6x16mm), AFA fiber with crimp-cap | Covaris, part # 520052 |
| [Optional - for starting material quality assessment] One of the following:<br>• Standard Sensitivity RNA Analysis Kit (20nt Lower Marker)<br>• Agilent RNA 6000 Nano Kit | • Advanced Analytical Technologies, part # DNF-489<br>• 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<br>(with programmable heated lid) | General lab supplier |
| One of the following:<br>• Fragment Analyzer Automated CE System<br>• 2100 Bioanalyzer Desktop System | • Advanced Analytical Technologies, part # FSv2-CE2 or FSv2-CE10<br>• 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 #<br>• 13500-890 (110 V/120 V) or<br>• 14216-214 (230 V) |
| Midi plate insert for heating system<br>Note: Two inserts are recommended to support successive heating procedures. | Illumina, catalog # BD-60-601 |
| Stroboscope | General lab supplier |
| One of the following:<br>Note: Two systems are recommended to support successive heating procedures.<br>• SciGene TruTemp Heating System<br>• Hybex Microsample Incubator | • Illumina, catalog # SC-60-503 (115 V) or SC-60-504 (220 V)<br>• 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 |
| BRP | Bind rRNA Plate |
| CAP | Clean Up ALP Plate |
| CCP | cDNA Clean Up Plate |

| Acronym | Definition |
|---|---|
| CPP | Clean Up PCR Plate |
| CTA | A-Tailing Control |
| CTE | End Repair Control |
| CTL | Ligation Control |
| DCT | Diluted Cluster Template |
| DFP | Depleted RNA Fragmentation Plate |
| ELB | Elution Buffer |
| EPH | Elute, Prime, Fragment High Mix |
| EPM | Enhanced PCR Mix |
| FSA | First Strand Synthesis Act D Mix |
| GRM | Globin Removal Mix |
| H/M/R | Human/Mouse/Rat |
| HS | High Sample |
| IEM | Illumina Experiment Manager |
| LIG | Ligation Mix |
| LRM | Local Run Manager |
| LS | Low Sample |
| PCR | Polymerase Chain Reaction |
| PDP | Pooled Dilution Plate |
| PMM | PCR Master Mix |
| PPC | PCR Primer Cocktail |
| RBB | rRNA Binding Buffer |
| RCP | RNA CleanUp Plate |
| RMB | rRNA Removal Mix - Bacteria |
| RME | rRNA Removal Mix - Epidemiology |
| RMY | rRNA Removal Mix - Yeast |
| RRB | rRNA Removal Beads |
| RRM | rRNA Removal Mix |
| RRM G | rRNA Removal Mix - Gold |
| RRM P | rRNA Removal Mix - Plant |
| RRP | rRNA Removal Plate |
| RSB | Resuspension Buffer |
| SMM | Second Strand Marking Master Mix |
| SPB | Sample Purification Beads |
| STL | Stop Ligation Buffer |
| TSP1 | Target Sample Plate |

# Alternate Fragmentation Protocols



## Introduction

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

Some studies require larger insert sizes, such as splice variant analysis studies. To vary the insert size of your library, see *Modify RNA Fragmentation Time for Intact RNA* on page 36.

It is not always possible to extract intact total RNA. For instance, RNA extracted from FFPE samples is typically degraded. To vary the fragmentation time for degraded RNA, see *Modify RNA Fragmentation Time for Degraded RNA* on page 37.

## Modify RNA Fragmentation Time for Intact RNA

To modify the fragmentation of the RNA to allow for longer RNA fragments, the time of fragmentation can be shortened. Fragmentation time is reduced during *Deplete and Fragment RNA* on page 10 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. X is determined by the length of RNA desired. A range of suggested times and sizes is described in Table 12.

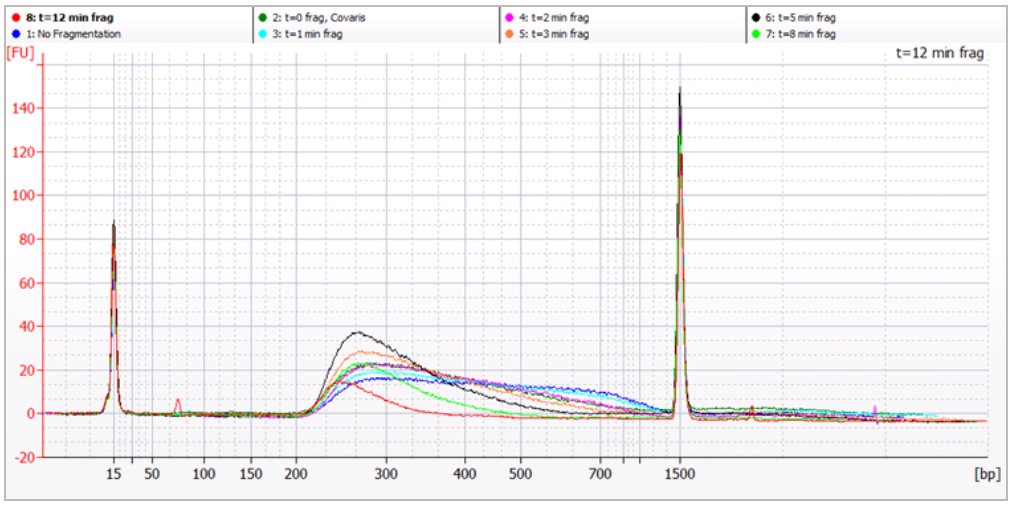
**Table 12 Library Insert Fragmentation Time**

| Time at 94°C (minutes) | Range of Insert Length[a] (bp) | Median Insert Length[a] (bp) | 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. Instead of a 94°C incubation, incubate at 65°C for 5 minutes, followed by a 4°C hold.This will elute the mRNA from the beads without fragmentation. The resulting cDNA fragments are smaller than the mRNA due to internal priming by the random hexamers in the EPH.

**Figure 12** 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.

## Modify RNA Fragmentation Time for Degraded RNA

For degraded RNA samples, adjust the fragmentation time to avoid over fragmentation of the RNA samples. The fragmentation time is reduced during *Deplete and Fragment RNA* on page 10 by either skipping or modifying the thermal cycler Elution 2-Frag-Prime program to 94°C for X minutes, followed by a 4°C hold.

Whether the samples undergo fragmentation and the amount of time used for fragmentation (X) is determined by the size range of the total RNA starting material. Perform the following steps to determine which fragmentation settings to use, if any.

1 Measure the size range of the total RNA starting material by running it on an Agilent RNA 6000 Nano or Pico chip.

2 Compare the resulting electropherogram to Figure 13–Figure 17, which show UHR that has been fragmented to various size ranges.

3 Determine which sample figure most resembles the size range of your starting material.

4 Use the thermal cycler settings recommended in the figure title of that size range to fragment your degraded RNA samples.

**Figure 13** Incubate Samples at 94°C for 8 Minutes, Followed By a 4°C Hold

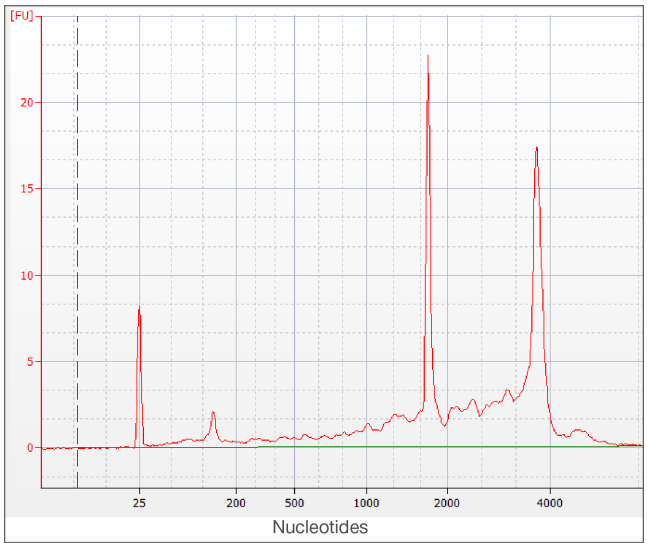



**Figure 14** Incubate Samples at 94°C for 7 Minutes, Followed By a 4°C Hold

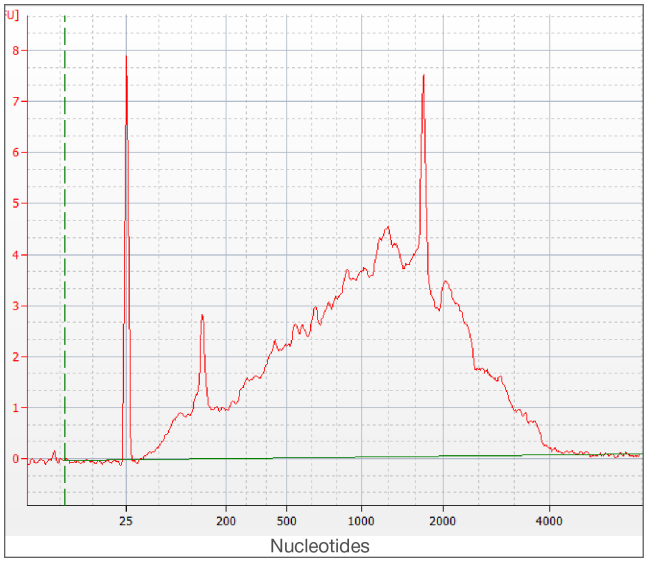


**Figure 15** Incubate Samples at 94°C for 6 Minutes, Followed By a 4°C Hold

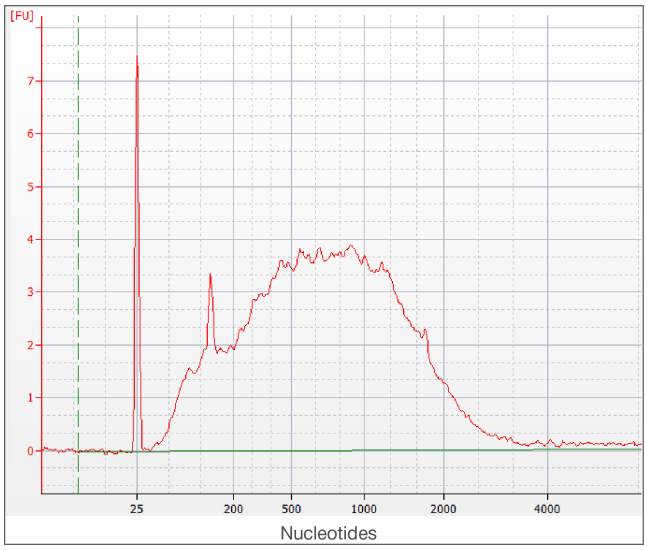
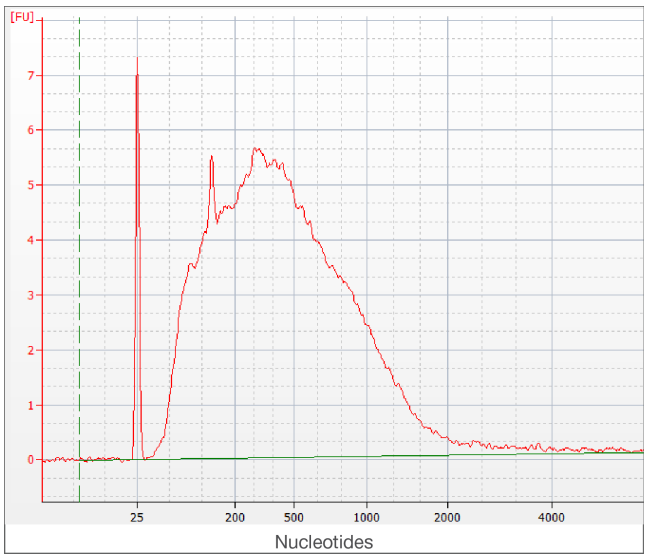
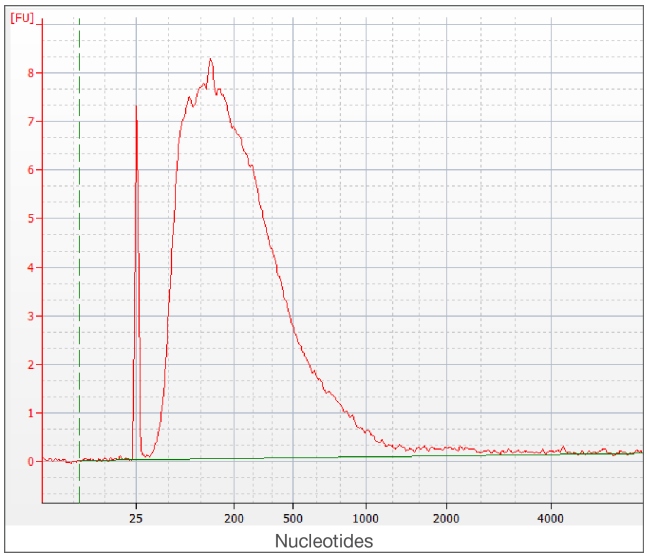



**Figure 16** Incubate Samples at 94°C for 4 Minutes, Followed By a 4°C Hold




For starting material smaller than shown in Figure 17, no fragmentation is necessary. Skip the thermal cycler Elution 2 -Frag -Prime program and proceed immediately to *Synthesize First Strand cDNA* on page 14.

**Figure 17** No Fragmentation Necessary.



# Technical Assistance

For technical assistance, contact Illumina Technical Support.

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**Safety data sheets (SDSs)**—Available on the Illumina website at support.illumina.com/sds.html.

**Product documentation**—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.

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