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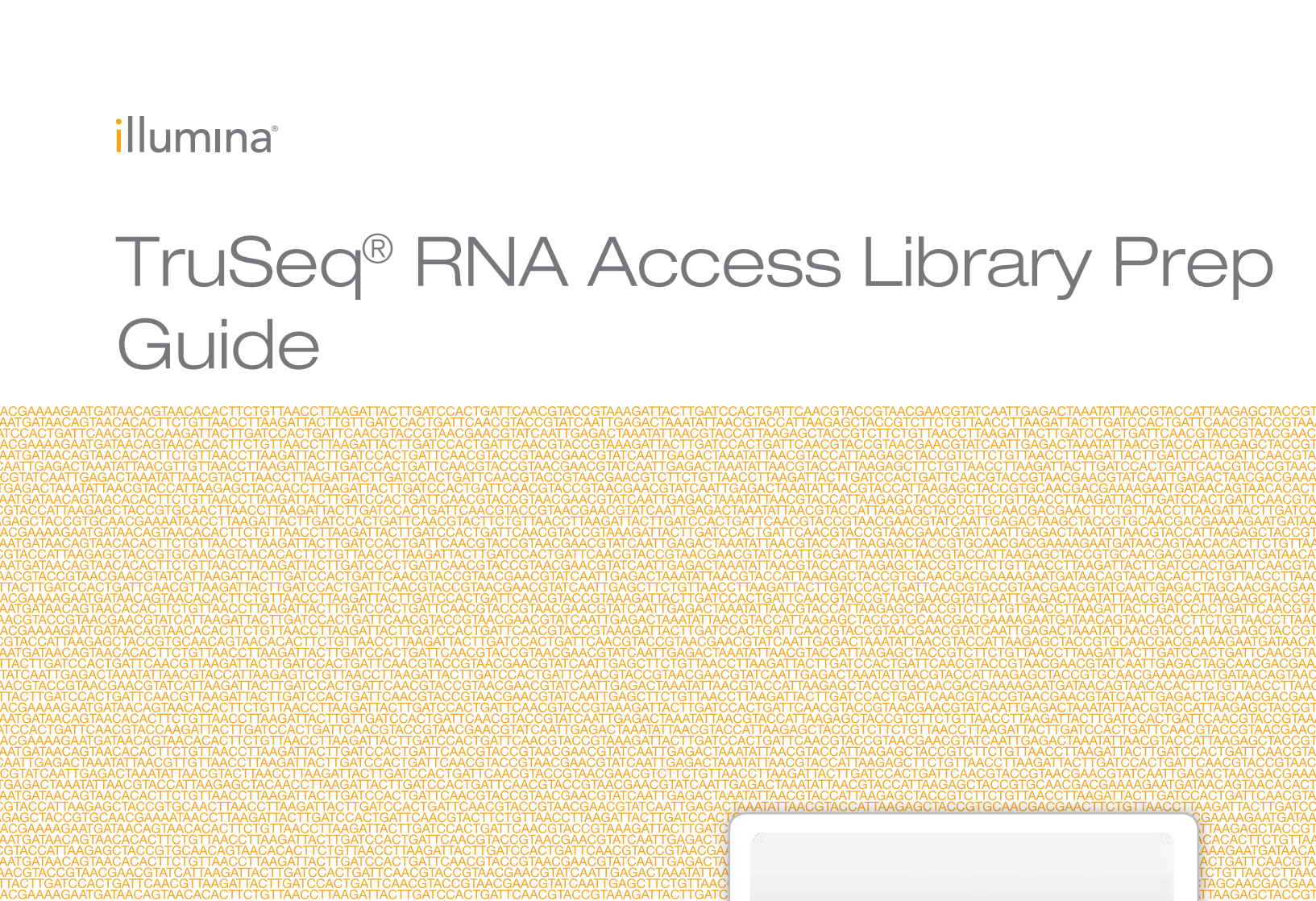
TruSeq® RNA Access Library Prep Guide



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RS-301-9001DOC
Part # 15049525 Rev. B
July 2014

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Revision History

Part #	Revision	Date	Description of Change

15049525	B	July 2014	• Removed instructions regarding barcodes and replaced with instructions to label plate with a pen
			• Modified the following plate names:
			  • NEH1 changed to RAH1 (RNA Access Hyb 1)
			  • NEW1 changed to RAW1 (RNA Access Wash 1)
			  • NEH2 changed to RAH2 (RNA Access Hyb 2)
			  • NEW2 changed to RAW2 (RNA Access Wash 2)
			  • NEC1 changed to RAC1 (RNA Access Clean Up 1)
			  • NEA changed to RAA (RNA Access Amplification)
			  • NEC2 changed to RAC2 (RNA Access Clean Up 2)
			  • NEL changed to RAL (RNA Access Library)
			• Replaced DEPC-treated water with nuclease-free water to *Fragment RNA*.
			• To *Clean Up ALP*
			  • Changed CAP plate Resuspension Buffer volume from 17.5 µl to 22.5 µl
			  • Changed final supernatant transfer volume from 15 µl to 20 µl
			• Included initial 98°C for 30 seconds setting in Preparation steps for *First PCR Amplification*.
			• Added AMPure XP beads to *Consumables and Equipment*
			• Removed distilled water and PCR-grade water from *Consumables and Equipment*

15049525	A	April 2014	Initial Release

Table of Contents

Overview

Chapter 1

Introduction

This protocol explains how to convert total RNA into a library of template molecules of known strand origin, then capture the coding regions of the transcriptome using the reagents provided in Illumina® TruSeq® RNA Access Library Prep kits. The resulting library is suitable for subsequent cluster generation and sequencing.

The RNA is fragmented into small pieces using divalent cations under elevated temperature. cDNA is generated from the cleaved RNA fragments using random priming during first and second strand synthesis and sequencing adapters are ligated to the resulting double-stranded cDNA fragments. The coding regions of the transcriptome are then captured from this library using sequence-specific probes to create the final library.

This library prep protocol offers:

- High data quality even from degraded or FFPE-derived RNA samples
- Input requirement as low as 10 ng for fresh/frozen samples and 20 ng for FFPE samples
- Uniform capture of the coding transcriptome, reducing sequencing requirement while maintaining discovery power
- Up to 24 unique indexes and 4-plex pre-enrichment pooling for the most efficient use of your sequencing read budget
- Strand information on RNA transcripts
- High throughput, automation-friendly procedures

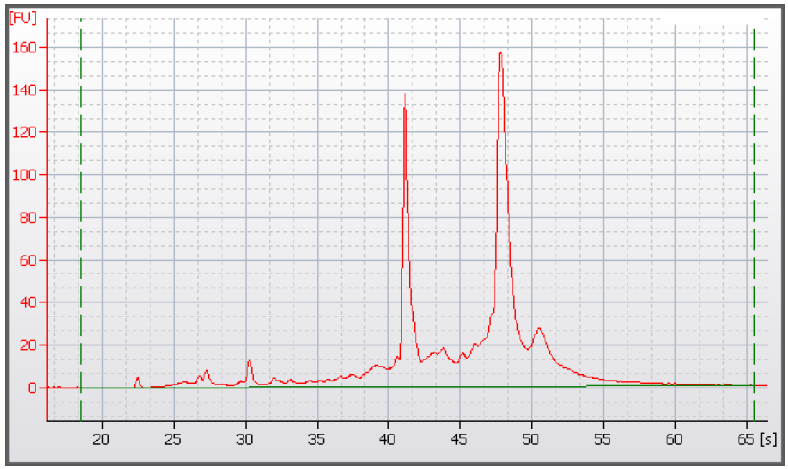
RNA Input Recommendations

It is important to follow the TruSeq RNA Access Library Prep input recommendations.

Total RNA Input

- This protocol is optimized for 10–100 ng of human total RNA.
  - Lower amounts might result in inefficient ligation and low yield.
- The protocol has been tested using 10 ng of high-quality universal human reference total RNA as input.
  - Use of RNA from other tissues or qualities might require further optimization regarding the initial input amount.
- It is important to know the quality of the RNA starting material. The fragmentation conditions were optimized for high-quality RNA. The following figure shows a Universal Human Reference (UHR) starting RNA Bioanalyzer trace.

Figure 1 Starting RNA Bioanalyzer Trace



- Degraded or FFPE RNAs are shorter than full length RNA.
  - RNA that has DNA contamination results in an underestimation of the amount of RNA used.

- If starting with FFPE RNA, the sample input amount is based on sample quality. Illumina recommends using the percentage of RNA fragments > 200 nt fragment distribution value (DV_{200}) as a reliable determinant of FFPE RNA quality.

Table 1 FFPE RNA Input Recommendations

Quality	DV_{200}	Input Requirement Per Reaction
High	> 70%	20 ng
Medium	50–70%	20–40 ng
Low	30–50%	40–100 ng
Too Degraded	< 30%	Not recommended

  - For successful library prep, Illumina recommends using an RNA isolation method that includes a reverse-crosslinking step and DNase1 treatment, such as the QIAGEN RNeasy FFPE Kit or QIAGEN AllPrep DNA/RNA FFPE Kit.
  - Illumina determines FFPE RNA concentration by Nanodrop.
  - For best performance on samples close to edge of a quality classification, err towards the higher end of the input recommendation.

NOTE

For more information, see the *Evaluating RNA Quality from FFPE Samples* tech note for TruSeq RNA Access Library Prep. See *Additional Resources* on page 7 for information on how to download the tech note from the Illumina website.

- The following are examples of high, medium, and low quality FFPE traces and a trace of FFPE quality that is not recommended for use for TruSeq RNA Access Library Prep.

Figure 2 Example: High Quality FFPE (DV_{200} = 77%)

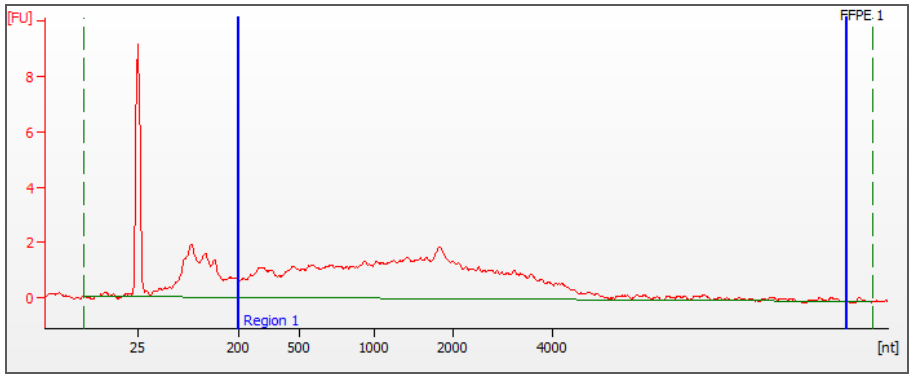


Figure 3 Example: Medium Quality FFPE (DV_{200} = 55%)

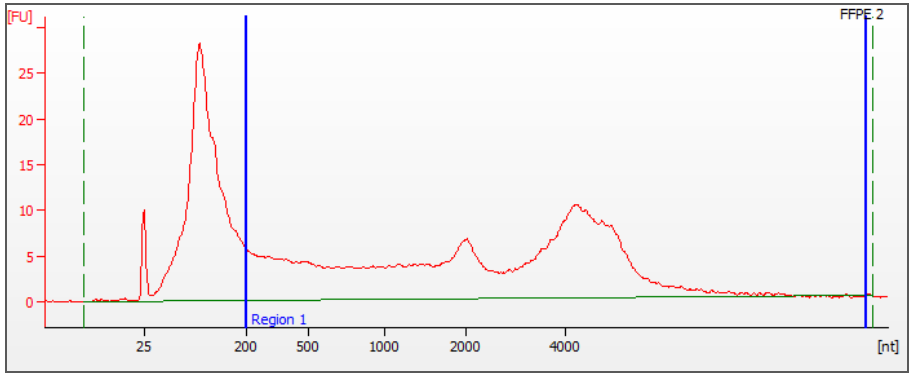


Figure 4 Example: Low Quality FFPE (DV_{200} = 30%)

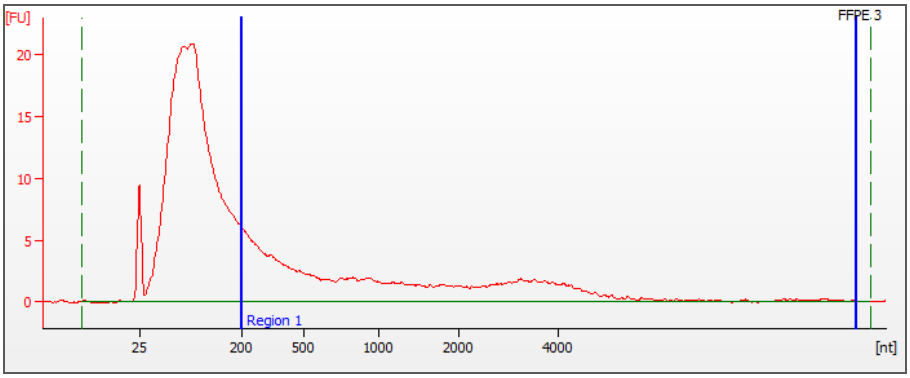
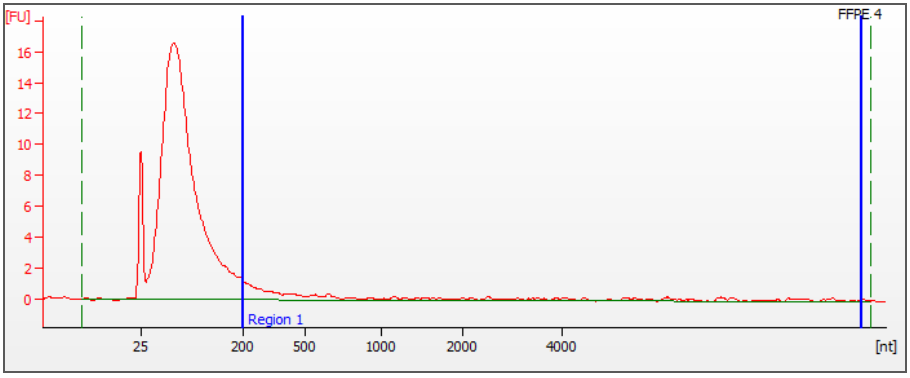


Figure 5 Example: FFPE Quality Not Recommended for Use (DV_{200} = 8%)



Positive Control

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

Additional Resources

The following resources are available for TruSeq RNA Access Library Prep protocol guidance and sample tracking. Access these and other resources on the Illumina website at support.illumina.com/sequencing/kits.ilmn. Then, select **TruSeq RNA Access Library Prep Kit Support**.

Resource	Description
Training	Illustrates elements of the TruSeq RNA Access Library Prep process. Viewing these videos is recommended for new and less experienced users before starting library prep. Click **Training** on **TruSeq RNA Access Library Prep Kit Support**
Best Practices	Provides best practices specific to this protocol. Review these best practices before starting library prep. Topics include: • Handling Liquids • Handling Master Mix Reagents • Handling Magnetic Beads • Avoiding Cross-Contamination • Potential DNA Contaminants • Temperature Considerations • Equipment Click **Best Practices** on **TruSeq RNA Access Library Prep Kit Support**
TruSeq RNA Access Library Prep Experienced User Card and Lab Tracking Form (part # 15049526)	Provides protocol instructions, but with less detail than what is provided in this user guide. **New or less experienced users are advised to follow this user guide and not the EUC and LTF.** Click **Documentation & Literature** on **TruSeq RNA Access Library Prep Kit Support**
Evaluating RNA Quality from FFPE Samples tech note	Provides effectivity profiles for FFPE RNA. Click **Documentation & Literature** on **TruSeq RNA Access Library Prep Kit Support**

Resource	Description
TruSeq Sample Preparation Pooling Guide (part # 15042173)	Provides TruSeq pooling guidelines for library prep. Review this guide before beginning library preparation. Click **Documentation & Literature** on **TruSeq RNA Access Library Prep Kit Support**
Sequencing Library qPCR Quantification Guide (part # 11322363)	Describes a qPCR method for quantifying sequencing by synthesis (SBS) libraries generated using the Illumina library prep protocols. Click **Documentation & Literature** on **TruSeq RNA Access Library Prep Kit Support**
Illumina Experiment Manager (IEM)	Enables you to create and edit appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate. To download the software, click **Downloads** on **TruSeq RNA Access Library Prep Kit Support** To download the documentation, click **Documentation & Literature** on **TruSeq RNA Access Library Prep Kit Support**
BaseSpace®	Sequencing data analysis tool that also enables you to organize samples, libraries, pools, and run in a single environment. For more information on BaseSpace see, support.illumina.com/sequencing/sequencing_software/basespace.ilmn

Protocol

Chapter 2

Introduction

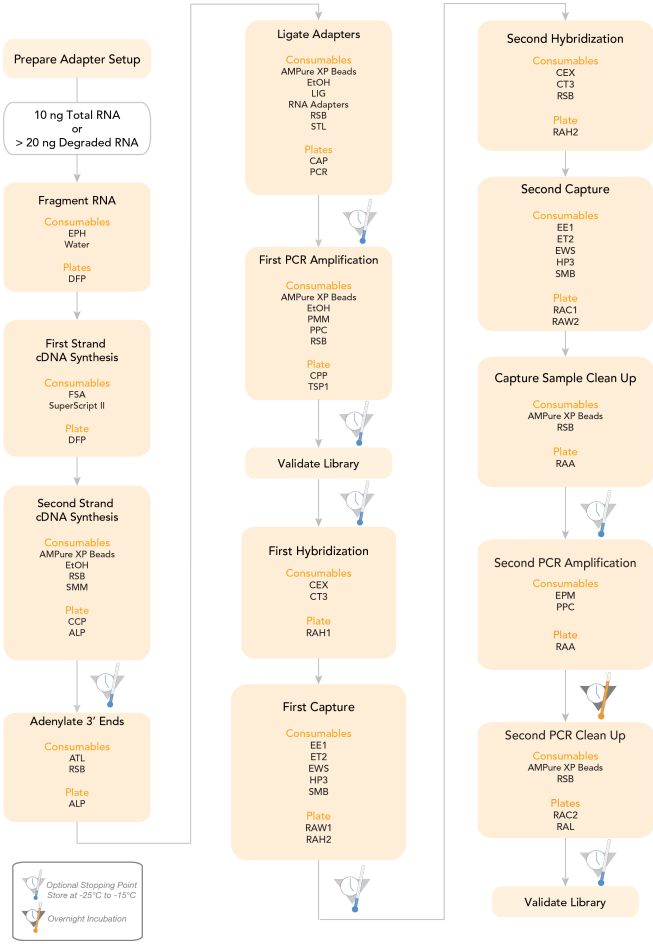
This chapter describes the TruSeq RNA Access Library Prep protocol.

- Review Best Practices before proceeding. See *Additional Resources* on page 7 for information on how to access TruSeq RNA Access Library Prep Best Practices on the Illumina website.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- If you are pooling, record information about your samples before beginning library prep for later use in data analysis.
  - Use IEM to create and edit sample sheets for Illumina sequencing systems and analysis software. See *Additional Resources* on page 7 for information on how to download IEM software and documentation from the Illumina website.
  - *TruSeq Sample Preparation Pooling Guide (part # 15042173)*—See *Additional Resources* on page 7 for information on how to download the guide from the Illumina website.
- Review Appendix A Supporting Information to confirm your kit contents and make sure that you have obtained all of the requisite equipment and consumables.

Library Prep Workflow

The following illustrates the processes of the TruSeq RNA Access Library Prep protocol to prepare templates using 24 indexed adapter tubes.

Figure 6 TruSeq RNA Access Library Prep Workflow



Prepare Adapter Setup

Use IEM or BaseSpace to record information about your samples before beginning library preparation.

- Do one of the following:
  - Use IEM to create and edit sample sheets for Illumina sequencing systems and analysis software. See *Additional Resources* on page 7 for information on how to download IEM software and documentation from the Illumina website.
  - Use BaseSpace to organize samples, libraries, pools, and a run for Illumina sequencing systems and analysis software. See *Additional Resources* on page 7 for information on how to access BaseSpace or download BaseSpace documentation from the Illumina website.
- Review planning steps in the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See *Additional Resources* on page 7 for information on how to download the guide from the Illumina website.

Illumina recommends arranging samples that will be combined into a common pool in the same row. Include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.

Fragment RNA

This process fragments and primes RNA for cDNA synthesis.

NOTE
If starting with FFPE RNA, the sample input amount is based on sample quality. Illumina recommends using the percentage of RNA fragments > 200 nt DV_{200} as a reliable determinant of FFPE RNA quality.

WARNING
If starting with FFPE RNA, do not perform the *Incubate 1 DFP* steps in this procedure.

Table 2 FFPE RNA Input Recommendations

Quality	DV_{200}	Input Requirement Per Reaction
High	> 70%	20 ng
Medium	50–70%	20–40 ng
Low	30–50%	40–100 ng
Too Degraded	< 30%	Not recommended

NOTE
For more information, see the *Evaluating RNA Quality from FFPE Samples* tech note for TruSeq RNA Access Library Prep. See *Additional Resources* on page 7 for information on how to download the tech note from the Illumina website.

Consumables

Item	Quantity	Storage	Supplied By
Elute, Prime, Fragment High Mix (EPH)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	-25°C to -15°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
Total RNA	10 ng fresh/frozen RNA per reaction or 20–100 ng FFPE RNA per reaction (see Table 2)	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	1	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	1	15°C to 30°C	User
Nuclease-free ultra pure water	Enough to dilute each total RNA sample to a final volume of 8.5 µl	15°C to 30°C	User

Preparation

- Remove the following from -25°C to -15°C storage and thaw them at room temperature:
  - Elute, Prime, Fragment High Mix
  - Resuspension Buffer

  NOTE
  The Resuspension Buffer can be stored at 2°C to 8°C after the initial thaw.
- Pre-program the thermal cycler with the following program and save as **Elution 2 - Frag - Prime**:
  - Choose the pre-heat lid option and set to 100°C
  - 94°C for 8 minutes, 4°C hold
- Set the centrifuge to 15°C to 25°C, if refrigerated.
- Label a new 96-well HSP plate **DFP** (Depleted RNA Fragmentation Plate) with a smudge resistant pen.

Make DFP

1. Dilute total RNA with nuclease-free ultra pure water to a final volume of 8.5 µl in each well of the new 96-well HSP plate labeled **DFP**.
2. Add 8.5 µl Elute, Prime, Fragment High Mix to each well of the DFP plate. Mix thoroughly as follows:
   a. Seal the DFP plate with a Microseal 'B' adhesive seal.
   b. Shake the DFP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.
3. Return the Elute, Prime, Fragment High Mix to -25°C to -15°C storage.

Incubate 1 DFP

WARNING
If starting with FFPE RNA, do not perform this incubation procedure. Proceed immediately to *Synthesize First Strand cDNA* on page 17.

1. Place the sealed DFP plate on the pre-programmed thermal cycler. Close the lid and select **Elution 2 - Frag - Prime** to fragment and prime the RNA.
   a. Choose the pre-heat lid option and set to 100°C
   b. 94°C for 8 minutes
   c. Hold at 4°C
2. Remove the DFP plate from the thermal cycler when it reaches 4°C and centrifuge briefly.
3. Proceed immediately to *Synthesize First Strand cDNA* on page 17.

Synthesize First Strand cDNA

This process reverse transcribes the cleaved RNA fragments that were primed with random hexamers into first strand cDNA using reverse transcriptase. The addition of actinomycin D to the First Stand Synthesis Act D mix (FSA) prevents spurious DNA-dependent synthesis, while allowing RNA-dependent synthesis, improving strand specificity.

Consumables

Item	Quantity	Storage	Supplied By
First Strand Synthesis Act D Mix (FSA)	1 tube	-25°C to -15°C	Illumina
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	1	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	1	15°C to 30°C	User
SuperScript II Reverse Transcriptase	1 tube	-25°C to -15°C	User

WARNING

First Strand Synthesis Act D Mix 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. Refer to the safety data sheet (SDS) for detailed environmental, health, and safety information. SDSs are available on the Illumina website at www.illumina.com/msds.

Preparation

- Remove one tube of First Strand Synthesis Act D Mix from -25°C to -15°C storage and thaw it at room temperature.

- Pre-program the thermal cycler with the following program and save as **Synthesize 1st Strand**:
  - Choose the pre-heat lid option and set to 100°C
  - 25°C for 10 minutes
  - 42°C for 15 minutes
  - 70°C for 15 minutes
  - Hold at 4°C
- Make sure that the microplate shaker is properly calibrated to 1000 rpm using a stroboscope.

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

Add FSA

1. Remove the adhesive seal from the DFP plate.
2. Centrifuge the thawed First Strand Synthesis Mix Act D tube at 600 × g for 5 seconds.
3. Add 50 µl SuperScript II to the First Strand Synthesis Act D Mix tube. Mix gently, but thoroughly and centrifuge briefly. If you are not using the entire contents of the First Strand Synthesis Act D Mix tube, add SuperScript II at a ratio of 1 µl SuperScript II for each 9 µl First Strand Synthesis Act D Mix.
   Label the First Strand Synthesis Mix Act D tube to indicate that the SuperScript II has been added.
4. Add 8 µl of First Strand Synthesis Mix Act D and SuperScript II mix to each well of the DFP plate. Mix thoroughly as follows:
   a. Seal the DFP plate with a Microseal 'B' adhesive seal.
   b. Shake the DFP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.
5. Return the First Strand Synthesis Mix Act D tube to -25°C to -15°C storage immediately after use.

Incubate 2 DFP

1. Place the sealed DFP plate on the pre-programmed thermal cycler. Close the lid and select **Synthesize 1st Strand**.
   a. Choose the pre-heat lid option and set to 100°C
   b. 25°C for 10 minutes
   c. 42°C for 15 minutes
   d. 70°C for 15 minutes
   e. Hold at 4°C
2. When the thermal cycler reaches 4°C, remove the DFP plate from the thermal cycler and proceed immediately to *Synthesize Second Strand cDNA* on page 20.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand, incorporating dUTP in place of dTTP to generate ds cDNA. The incorporation of dUTP quenches the second strand during amplification, because the polymerase does not incorporate past this nucleotide. AMPure XP beads are used to separate the ds cDNA from the second strand reaction mix. At the end of this process, you have blunt-ended cDNA.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Second Strand Marking Master Mix (SMM)	1 tube per 48 reactions	-25°C to -15°C	Illumina
96-well MIDI plates	2	15°C to 30°C	User
AMPure XP beads	90 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	4	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- Remove the Second Strand Marking Master Mix from -25°C to -15°C storage and thaw it at room temperature.
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.

- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 7 for information on how to access TruSeq RNA Access Library Prep Best Practices on the Illumina website.
- Pre-heat the thermal cycler to 16°C.
- Choose the thermal cycler pre-heat lid option and set the lid to 30°C
- Label a new 96-well MIDI plate **ALP** (Adapter Ligation Plate) with a smudge resistant pen.
- Label a new 96-well MIDI plate **CCP** (cDNA Clean Up Plate) with a smudge resistant pen.

Add SMM

1. Remove the adhesive seal from the DFP plate.
2. Add 5 µl of Resuspension Buffer to each well of the DFP plate.
3. Centrifuge the thawed Second Strand Marking Master Mix at 600 × g for 5 seconds.
4. Add 20 µl of thawed Second Strand Marking Master Mix to each well of the DFP plate. Mix thoroughly as follows:
   a. Seal the DFP plate with a Microseal 'B' adhesive seal.
   b. Shake the DFP plate on a microplate shaker continuously at 1600 rpm for 20 seconds.
5. Return the Second Strand Marking Master Mix tube to -25°C to -15°C storage after use.

Incubate 3 DFP

1. Place the sealed DFP plate on the pre-heated thermal cycler. Close the lid and incubate at 16°C for 1 hour.
2. Remove the DFP plate from the thermal cycler and place it on the bench.
3. Remove the adhesive seal from the DFP plate.
4. Let the DFP plate stand to bring it to room temperature.

Clean Up DFP

1. Vortex the AMPure XP beads until they are well dispersed.
2. Add 90 µl of well-mixed AMPure XP beads to each well of the new MIDI plate labeled **CCP**.
3. Transfer the entire contents from each well of the DFP plate to the corresponding well of the CCP plate containing AMPure XP beads. Mix thoroughly as follows:
   a. Seal the CCP plate with a Microseal 'B' adhesive seal.
   b. Shake the CCP plate on a microplate shaker at 1800 rpm for 2 minutes.
4. Incubate the CCP plate at room temperature for 5 minutes.
5. Centrifuge the CCP plate at 280 × g for 1 minute.
6. Remove the adhesive seal from the CCP plate.
7. Place the CCP plate on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells.
8. Remove and discard 135 µl supernatant from each well of the CCP plate.

   NOTE
   Leave the CCP plate on the magnetic stand while performing the following 80% EtOH wash steps (9–11).

9. With the CCP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.
10. Incubate the CCP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well.
11. Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.
12. Let the CCP plate stand at room temperature for 5 minutes to dry, and then remove the CCP plate from the magnetic stand.
13. Centrifuge the thawed, room temperature Resuspension Buffer at 600 × g for 5 seconds.
14. Add 17.5 µl Resuspension Buffer to each well of the CCP plate. Mix thoroughly as follows:
    a. Seal the CCP plate with a Microseal 'B' adhesive seal.
    b. Shake the CCP plate on a microplate shaker at 1800 rpm for 2 minutes.

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

16 Centrifuge the CCP plate at 280 × g for 1 minute.

17 Remove the adhesive seal from the CCP plate.

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

19 Transfer 15 µl supernatant (ds cDNA) from the CCP plate to the new MIDI plate labeled **ALP**.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Adenylate 3' Ends* on page 24, you can safely stop the protocol here. If you are stopping, seal the ALP plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Adenylate 3' Ends

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

Consumables

Item	Quantity	Storage	Supplied By
A-Tailing Mix (ATL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	3	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	3	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the A-Tailing Mix from -25°C to -15°C storage. Thaw it at room temperature and then place it on ice.
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.

- Remove the ALP plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up DFP* on page 22.
  - Let it thaw at room temperature.
  - Centrifuge the thawed ALP plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the ALP plate.
- Pre-heat two microheating systems: system 1 to 37°C and system 2 to 70°C.

Add ATL

1. Centrifuge the thawed A-Tailing Mix tube at 600 × g for 5 seconds.
2. Add 2.5 µl Resuspension Buffer to each well of the ALP plate.
3. Add 12.5 µl thawed A-Tailing Mix to each well of the ALP plate. Mix thoroughly as follows:
   a. Seal the ALP plate with a Microseal 'B' adhesive seal.
   b. Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.
4. Centrifuge the ALP plate at 280 × g for 1 minute.
5. Return the A-Tailing Mix tube to -25°C to -15°C storage.

Incubate 1 ALP

1. Place the sealed ALP plate on the pre-heated microheating system 1. Close the lid and incubate at 37°C for 30 minutes.
2. Immediately after the 37°C incubation, remove the ALP plate from system 1 and place the plate on the pre-heated microheating system 2. Close the lid and incubate at 70°C for 5 minutes.
3. Set the microheating system 1 to 30°C in preparation for *Ligate Adapters*.
4. Immediately remove the ALP plate from the microheating system 2 and place the plate on ice for 1 minute.
5. Proceed immediately to *Ligate Adapters* on page 26.

Ligate Adapters

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

Consumables

Item	Quantity	Storage	Supplied By
RNA Adapter Indexes (AR001–AR016, AR018–AR023, AR025, AR027)	1 tube of each index being used, per column of 8 reactions	-25°C to -15°C	Illumina
Ligation Mix (LIG)	1 tube per 48 reactions	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Stop Ligation Buffer (STL)	1 tube per 48 reactions	-25°C to -15°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
AMPure XP beads	92 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	800 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	7	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	4–28	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	4–28	15°C to 30°C	User

Preparation

- Remove the following from -25°C to -15°C storage and thaw them at room temperature:
  - RNA Adapter tubes (depending on the RNA Adapter Indexes being used).

    NOTE
    - Review the *TruSeq Sample Preparation Pooling Guide (part # 15042173)*. See for information on how to download the guide from the Illumina website.
    - When indexing libraries using adapter index tubes, Illumina recommends arranging samples that are going to be combined into a common pool in the same row. Also, include a common index in each column. This arrangement facilitates pipetting operations when dispensing indexed adapters and pooling indexed libraries later in the protocol.

  - Stop Ligation Buffer

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

- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Review Best Practices for *Handling Magnetic Beads*. See *Additional Resources* on page 7 for information on how to access TruSeq RNA Access Library Prep Best Practices on the Illumina website.
- Pre-heat the microheating system 1 to 30°C.
- Label a new 96-well MIDI plate **CAP** (Clean Up ALP Plate) with a smudge resistant pen.
- Label a new 96-well HSP plate **PCR** (Polymerase Chain Reaction Plate) with a smudge resistant pen.

Add LIG

1. Centrifuge the thawed RNA Adapter tubes at 600 × g for 5 seconds.
2. Immediately before use, remove the Ligation Mix tube from -25°C to -15°C storage.
3. Remove the adhesive seal from the ALP plate.
4. Add 2.5 µl Resuspension Buffer to each well of the ALP plate.

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

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

7 Add 2.5 µl thawed RNA Adapter Index to each well of the ALP plate.

8 Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

9 Centrifuge the ALP plate at 280 × g for 1 minute.

Incubate 2 ALP

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

2 Remove the ALP plate from the microheating system.

Add STL

1 Remove the adhesive seal from the ALP plate.

2 Add 5 µl Stop Ligation Buffer to each well of the ALP plate to inactivate the ligation mix. Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

3 Centrifuge the ALP plate at 280 × g for 1 minute.

Clean Up ALP

1 Remove the adhesive seal from the ALP plate.

2 Vortex the AMPure XP beads for at least 1 minute or until they are well dispersed.

3 Add 42 µl mixed AMPure XP beads to each well of the ALP plate. Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

4 Incubate the ALP plate at room temperature for 5 minutes.

5 Centrifuge the ALP plate at 280 × g for 1 minute.

6 Remove the adhesive seal from the ALP plate.

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

8 Remove and discard 79.5 µl of supernatant from each well of the ALP plate. Take care not to disturb the beads.

NOTE
Leave the ALP plate on the magnetic stand while performing the following 80% EtOH wash steps (9–11).

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

10 Incubate the ALP plate at room temperature for 30 seconds, and then remove and discard all of the supernatant from each well. Take care not to disturb the beads.

11 Repeat steps 9 and 10 one time for a total of two 80% EtOH washes.

12 With the ALP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes.

13 Remove the ALP plate from the magnetic stand.

14 Add 52.5 µl Resuspension Buffer to each well of the ALP plate. Mix thoroughly as follows:
   a Seal the ALP plate with a Microseal 'B' adhesive seal.
   b Shake the ALP plate on a microplate shaker at 1800 rpm for 2 minutes.

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

16 Centrifuge the ALP plate at 280 × g for 1 minute.

17 Remove the adhesive seal from the ALP plate.

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

19 Transfer 50 µl of supernatant from each well of the ALP plate to the corresponding well of the new MIDI plate labeled **CAP**. Take care not to disturb the beads.

20 Vortex the AMPure XP beads until they are well dispersed.

21 Add 50 µl mixed AMPure XP beads to each well of the CAP plate for a second cleanup. Mix thoroughly as follows:
   a Seal the CAP plate with a Microseal 'B' adhesive seal.
   b Shake the CAP plate on a microplate shaker at 1800 rpm for 2 minutes.

22 Incubate the CAP plate at room temperature for 5 minutes.

23 Centrifuge the CAP plate at 280 × g for 1 minute.

24 Remove the adhesive seal from the CAP plate.

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

26 Remove and discard 95 µl of supernatant from each well of the CAP plate. Take care not to disturb the beads.

NOTE
Leave the CAP plate on the magnetic stand while performing the following 80% EtOH wash steps (27–29)

27 With the CAP plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well. Take care not to disturb the beads.

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

29 Repeat steps 27 and 28 one time for a total of two 80% EtOH washes.

30 With the CAP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes.

31 Remove the CAP plate from the magnetic stand.

32 Add 22.5 µl Resuspension Buffer to each well of the CAP plate. Mix thoroughly as follows:
   a Seal the CAP plate with a Microseal 'B' adhesive seal.
   b Shake the CAP plate on a microplate shaker at 1800 rpm for 2 minutes.

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

34 Centrifuge the CAP plate at 280 × g for 1 minute.

35 Remove the adhesive seal from the CAP plate.

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

37 Transfer 20 µl of supernatant from each well of the CAP plate to the corresponding well of the new HSP plate labeled **PCR**. Take care not to disturb the beads.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *First PCR Amplification* on page 32, you can safely stop the protocol here. If you are stopping, seal the PCR plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

First PCR Amplification

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

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

Consumables

Item	Quantity	Storage	Supplied By
PCR Master Mix (PMM)	1 tube per 48 reactions	-25°C to -15°C	Illumina
PCR Primer Cocktail (PPC)		-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
AMPure XP beads	50 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Ice bucket	As needed	-25°C to -15°C	User
Microseal 'A' film	1	15°C to 30°C	User
Microseal 'B' adhesive seals	3	15°C to 30°C	User

Item	Quantity	Storage	Supplied By
RNase/DNase-free eight-tube strips and caps (if using multichannel pipettes)	5	15°C to 30°C	User
RNase/DNase-free reagent reservoirs (if using multichannel pipettes)	5	15°C to 30°C	User

Preparation

- Prepare an ice bucket.
- Remove the PCR Master Mix and PCR Primer Cocktail from -25°C to -15°C storage. Thaw them at room temperature and then place them on ice.
- Centrifuge the thawed PCR Master Mix and PCR Primer Cocktail at 600 × g for 5 seconds.
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the AMPure XP beads from 2°C to 8°C storage and let stand for at least 30 minutes to bring them to room temperature.
- Remove the PCR plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up ALP* on page 28.
  - Let it thaw at room temperature.
  - Centrifuge the thawed PCR plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed PCR plate.
- Pre-program the thermal cycler with the following program and save as **PCR**:
  - Choose the pre-heat lid option and set to 100°C
  - 98°C for 30 seconds
  - 15 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 4°C

- Label a new 96-well MIDI plate **CPP** (Clean Up PCR Plate) with a smudge resistant pen.
- Label a new 96-well HSP plate **TSP1** (Target Sample Plate) with a smudge resistant pen.

Make PCR

1. Add 5 µl thawed PCR Primer Cocktail to each well of the PCR plate.
2. Add 25 µl thawed PCR Master Mix to each well of the PCR plate.
   a. Seal the PCR plate with a Microseal 'A' film.

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

   b. Shake the PCR plate on a microplate shaker at 1600 rpm for 20 seconds.
3. Centrifuge the PCR plate at 280 × g for 1 minute.
4. Return the PCR Primer Cocktail and Enhanced PCR Mix tubes to -25°C to -15°C storage.

Amp PCR

1. Place the sealed PCR plate on the pre-programmed thermal cycler. Close the lid, then select and run **PCR** to amplify the plate.
   a. Choose the pre-heat lid option and set to 100°C
   b. 98°C for 30 seconds
   c. 15 cycles of:
      — 98°C for 10 seconds
      — 60°C for 30 seconds
      — 72°C for 30 seconds
   d. 72°C for 5 minutes
   e. Hold at 4°C

Clean Up PCR

1. Remove the adhesive seal from the PCR plate.

2 Vortex the AMPure XP beads for at least 1 minute or until they are well dispersed.

3 Add 50 µl mixed AMPure XP beads to each well of the new MIDI plate labeled **CPP**.

4 Transfer the entire contents from each well of the PCR plate to the corresponding well of the CPP plate containing 50 µl mixed AMPure XP beads. Mix thoroughly as follows:
   a Seal the CPP plate with a Microseal 'B' adhesive seal.
   b Shake the CPP plate on a microplate shaker at 1800 rpm for 2 minutes.

5 Incubate the CPP plate at room temperature for 5 minutes.

6 Centrifuge the CPP plate at 280 × g for 1 minute.

7 Remove the adhesive seal from the CPP plate.

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

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

NOTE
Leave the CPP plate on the magnetic stand while performing the following 80% EtOH wash steps (10–12).

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

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

12 Repeat steps 10 and 11 one time for a total of two 80% EtOH washes.

13 With the CPP plate on the magnetic stand, let the samples air-dry at room temperature for 5 minutes, and then remove the plate from the magnetic stand.

14 Add 17.5 µl Resuspension Buffer to each well of the CPP plate. Make sure the Resuspension Buffer runs over the beads. Mix thoroughly as follows:
   a Seal the CPP plate with a Microseal 'B' adhesive seal.
   b Shake the CPP plate on a microplate shaker at 1800 rpm for 2 minutes.

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

16 Centrifuge the CPP plate at 280 × g for 1 minute.

17 Remove the adhesive seal from the CPP plate.

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

19 Transfer 15 µl of the clear supernatant from each well of the CPP plate to the corresponding well of the new HSP plate labeled **TSP1**.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Validate Library* on page 37, you can safely stop the protocol here. If you are stopping, seal the TSP1 plate with a Microseal 'B' adhesive seal and store at -25°C to -15°C for up to 7 days.

Validate Library

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

Quantify Library

Quantify your library using an Advanced Analytical Fragment Analyzer or Agilent Technologies 2100 Bioanalyzer. As an alternative, quantify using PicoGreen.

Quality Control

1 Do one of the following:
- Dilute 1 µl of resuspended construct with 1 µl Resuspension Buffer and load on an Advanced Analytical Fragment Analyzer using Standard Sensitivity NGS Fragment Analysis Kit.
- Load 1 µl of resuspended construct on an Agilent Technologies 2100 Bioanalyzer using a DNA-specific chip such as the Agilent DNA 1000.

2 Check the size and purity of the sample. The final product should be a band at approximately 260 bp.

Figure 7 Example of TruSeq RNA Access Library Prep Library Size Distribution

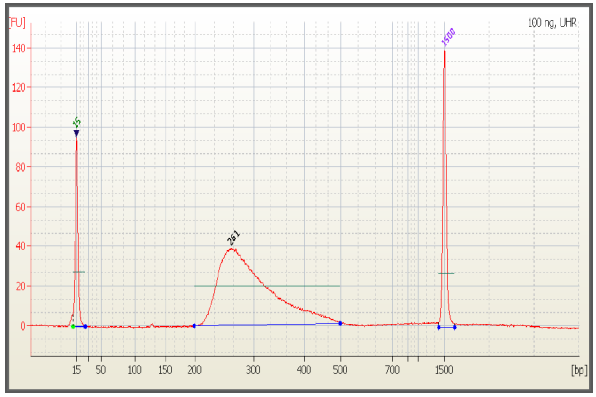
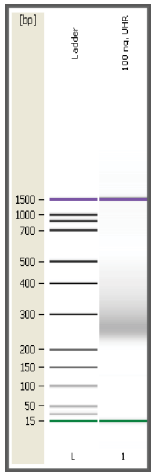


Figure 8 TruSeq RNA Access Library Prep 260 bp PCR Product



First Hybridization

This process mixes the DNA library with capture probes to targeted regions of interest. The recommended hybridization time makes sure that targeted regions bind to the capture probes thoroughly. This process also describes how to combine multiple libraries with different indexes into a single pool before enrichment.

Consumables

Item	Quantity	Storage	Supplied By
Capture Target Buffer 3 (CT3)	1 tube	-25°C to -15°C	Illumina
Coding Exome Oligos (CEX)	1 tube	-25°C to -15°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User
RNase/DNase-free eight-tube strips and caps (for multi-sample processing)	2	15°C to 30°C	User
[Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa)	1 per pooled sample	15°C to 30°C	User

Preparation

- Remove the following from -25°C to -15°C storage and thaw them at room temperature:
  - Capture Target Buffer 3
  - Coding Exome Oligos
- For multi-sample processing:
  - Use a multichannel pipette.
  - Distribute the Capture Target Buffer 3 and Coding Exome Oligos into separate eight-tube strips, dispensing equal volumes into each of the wells.

- Remove the TSP1 plate from -25°C to -15°C storage, if it was stored at the conclusion of *Clean Up PCR* and thaw on ice.
  - Centrifuge the thawed TSP1 plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed TSP1 plate.
- Pre-program the thermal cycler with the following program and save as **RNA HYB**:
  a Choose the pre-heat lid option and set to 100°C
  b 95°C for 10 minutes
  c 18 cycles of 1 minute incubations, starting at 94°C, then decreasing 2°C per cycle
  d 58°C for 90 minutes

  NOTE
  A 90 minute incubation is optimal for hybridization. Hybridizing longer than 2 hours results in a high degree of non-specific binding.
- Label a new 96-well HSP plate **RAH1** (RNA Access Hyb 1) with a smudge resistant pen.

Pool Libraries

Combine 200 ng of each DNA library for pooling.

NOTE
- The TruSeq RNA Access contains enough of each reagent for 4-plex pooling. Pooling at less than 4-plex results in the inability to process the number of samples supported by the kit.
- Illumina does not recommend pooling more than 4 samples.
- Pooling an odd number of samples does not affect data. For example, for 5 samples, you can pool 2 samples and 3 samples or 4 samples and 1 sample.

Table 3 DNA Libraries for Enrichment

Library Pool Complexity	Total DNA Library Mass (ng)
1-plex	200
2-plex	400
3-plex	600
4-plex	800

- If the total volume is greater than 45 µl, concentrate the pooled sample. Use either a vacuum concentrator or Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) according to manufacturer instructions.
  - If you are using a vacuum concentrator, Illumina recommends concentrating samples with a no heat and medium drying rate setting.
  - If you are using an Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa), it is not required to pre-rinse the device before use. Most of the volume filters through in 5 minutes, but up to 30 minutes can be required, depending on the starting volume.
- If the pooled sample volume after concentrating is less than 45 µl, bring the volume up to 45 µl with Resuspension Buffer.

Procedure

1. Thoroughly vortex the Capture Target Buffer 3 tube until the solution is completely resuspended. Visually make sure that no crystal structures are present.

   NOTE
   If crystals and cloudiness are observed, vortex the Capture Target Buffer 3 tube until it is clear.

2. Add the following reagents in the order listed to each well of the new 96-well HSP plate labeled **RAH1**:

Reagent	Volume (µl)
DNA library sample or library pool from TSP1 plate	45
Capture Target Buffer 3	50
Coding Exome Oligos	5
Total Volume per Sample	**100**

3. Mix thoroughly as follows:
   a. Seal the RAH1 plate with a Microseal 'B' adhesive seal. Make sure that the plate is tightly sealed to prevent potential evaporation. Use an adhesive seal roller to apply force to the seal and make sure that the seal is secured.
   b. Shake the RAH1 plate on a microplate shaker at 1200 rpm for 1 minute.
4. Centrifuge the RAH1 plate at 280 × g for 1 minute.

5 Place the sealed RAH1 plate on the pre-programmed thermal cycler. Close the lid, then select and run the **RNA HYB** program.

a Choose the pre-heat lid option and set to 100°C

b 95°C for 10 minutes

c 18 cycles of 1 minute incubations, starting at 94°C, then decreasing 2°C per cycle

d 58°C for forever

NOTE

Run the 58°C hybridization for 90 minutes. The RNA HYB program is set to 58°C for forever to make sure that the sample is at 58°C when the plate is removed.

6 Immediately remove the plate from the thermal cycler after 90 minutes of 58°C hybridization and proceed immediately to *First Capture* on page 43.

NOTE

The total run time of the RNA HYB program is approximately 2 hours. Hybridizing longer than 2 hours results in a high degree of non-specific binding.

First Capture

This process uses streptavidin beads to capture probes hybridized to the targeted regions of interest. Two heated wash procedures remove non-specific binding from the beads. The enriched library is then eluted from the beads and prepared for a second round of hybridization.

Consumables

Item	Quantity	Storage	Supplied By
2N NaOH (HP3)	1 tube	-25°C to -15°C	Illumina
Elute Target Buffer 2 (ET2)	1 tube	2°C to 8°C	Illumina
Enrichment Elution Buffer 1 (EE1)	1 tube	-25°C to -15°C	Illumina
Enrichment Wash Solution (EWS)	1 tube	-25°C to -15°C	Illumina
Streptavidin Magnetic Beads (SMB)	1 tube	2°C to 8°C	Illumina
1.7 ml microcentrifuge tube	1	15°C to 30°C	User
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
Microseal 'B' adhesive seals	6	15°C to 30°C	User

Preparation

- Remove the 2N NaOH, Enrichment Elution Buffer 1, and Enrichment Wash Solution from -25°C to -15°C storage and thaw at room temperature.
- Remove the Elute Target Buffer 2 and Streptavidin Magnetic Beads from 2°C to 8°C storage and let stand at room temperature.
- Pre-heat the microheating system to 50°C.

- Label a new 96-well MIDI plate **RAW1** (RNA Access Wash 1) with a smudge resistant pen.
- Label a new 96-well HSP plate **RAH2** (RNA Access Hyb 2) with a smudge resistant pen.

First Bind

1. Remove the RAH1 plate from the thermal cycler.
2. Centrifuge the RAH1 plate at 280 × g for 1 minute.
3. Remove the adhesive seal from the RAH1 plate. Take care when removing the seal to avoid spilling the contents of the wells.
4. Transfer the entire contents (~100 µl) from each well of the RAH1 plate to the corresponding well of the new 96-well MIDI plate labeled **RAW1**.
5. Vortex the Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 µl well-mixed Streptavidin Magnetic Beads to the wells of the RAW1 plate.
6. Mix thoroughly as follows:
   a. Seal the RAW1 plate with a Microseal 'B' adhesive seal.
   b. Shake the RAW1 plate on a microplate shaker at 1200 rpm for 5 minutes.
7. Let the RAW1 plate stand at room temperature for 25 minutes.
8. Centrifuge the RAW1 plate at 280 × g for 1 minute.
9. Remove the adhesive seal from the RAW1 plate.
10. Place the RAW1 plate on the magnetic stand for 2 minutes at room temperature or until the liquid is clear.
11. Carefully remove and discard all of the supernatant from each well of the RAW1 plate without disturbing the beads.
12. Remove the RAW1 plate from the magnetic stand.

First Wash

1. Make sure that the Enrichment Wash Solution tube is at room temperature, then thoroughly vortex the tube.

   NOTE
   It is normal that the Enrichment Wash Solution can be cloudy after vortexing.

2. Add 200 µl Enrichment Wash Solution to each well of the RAW1 plate.
3. Mix thoroughly as follows:
   a. Seal the RAW1 plate with a Microseal 'B' adhesive seal.
   b. Shake the RAW1 plate on a microplate shaker at 1800 rpm for 4 minutes.
   c. Remove the adhesive seal from the RAW1 plate.
   d. Gently pipette the entire volume of each well up and down to ensure complete resuspension of the sample.
4. Seal the RAW1 plate with a Microseal 'B' adhesive seal.
5. Place the sealed RAW1 plate on the **pre-heated** microheating system. Close the lid and incubate at 50°C for 20 minutes.
6. Place the magnetic stand next to the microheating system for immediate access.
7. Remove the RAW1 plate from the microheating system and ***immediately*** place it on the magnetic stand for 2 minutes or until the liquid is clear.
8. Remove the adhesive seal from the RAW1 plate.
9. Immediately remove and discard all of the supernatant from each well of the RAW1 plate.
10. Remove the RAW1 plate from the magnetic stand.
11. Repeat steps 2–10 one time for a total of two Enrichment Wash Solution washes.

First Elution

1 Add the following reagents in the order listed in a new 1.7 ml microcentrifuge tube to create the elution pre-mix. Multiply each volume by the number of pooled samples being prepared. The volumes include an excess amount for processing multiple samples.

Reagent	Volume (µl)
Enrichment Elution Buffer 1	28.5
2N NaOH	1.5
Total volume per enrichment	**30**

2 Vortex the elution pre-mix tube, then add **23** µl of the mix to each well of the RAW1 plate.

3 Mix thoroughly as follows:
   a Seal the RAW1 plate with a Microseal 'B' adhesive seal.
   b Shake the RAW1 plate on a microplate shaker at 1800 rpm for 2 minutes.

4 Let the RAW1 plate stand at room temperature for 2 minutes.

5 Centrifuge the RAW1 plate at 280 × g for 1 minute.

6 Carefully remove the adhesive seal from the RAW1 plate to avoid spilling the contents of the wells.

7 Place the RAW1 plate on the magnetic stand for 2 minutes or until the liquid is clear.

8 Transfer 21 µl of clear supernatant from each well of the RAW1 plate to the corresponding well of the new HSP plate labeled **RAH2**. Take care not to disturb the beads.

NOTE
Illumina recommends using a 20 µl single channel or multichannel pipette set to 10.5 µl to perform two consecutive transfers of 10.5 µl. This technique reduces sample loss by making sure that all of the liquid is transferred without disturbing the beads.

9 Add 4 µl Elute Target Buffer 2 to each well of the RAH2 plate containing samples to neutralize the elution.

10 Mix thoroughly as follows:
   a Seal the RAH2 plate with a Microseal 'B' adhesive seal.
   b Shake the RAH2 plate on a microplate shaker at 1200 rpm for 1 minute.

11 Centrifuge the RAH2 plate at 280 × g for 1 minute.

12 Store the remaining reagents as follows:
   a Place the Elute Target Buffer 2 and Streptavidin Magnetic Beads tubes in 2°C to 8°C storage.
   b Place the 2N NaOH, Enrichment Elution Buffer 1, and Enrichment Wash Solution tubes in -25°C to -15°C storage.
   c Discard any remaining elution pre-mix.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Second Hybridization* on page 48, you can safely stop the protocol here. If you are stopping, seal the RAH2 plate with a Microseal 'B' adhesive seal and store it at -25°C to -15°C for up to 7 days.

Second Hybridization

This process combines the eluted DNA library from the first enrichment round with additional capture probes to targeted regions of interest. This second hybridization is required to ensure high specificity of the captured regions.

Consumables

Item	Quantity	Storage	Supplied By
Capture Target Buffer 3 (CT3)	1 tube	-25°C to -15°C	Illumina
Coding Exome Oligos (CEX)	1 tube	-25°C to -15°C	Illumina
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
Microseal 'B' adhesive seal	1	15°C to 30°C	User

Preparation

- Remove the following from -25°C to -15°C storage and thaw them at room temperature:
  - Capture Target Buffer 3
  - Coding Exome Oligos
- Remove the Resuspension Buffer from 2°C to 8°C storage and bring it to room temperature.
- Remove the RAH2 plate from -25°C to -15°C storage, if it was stored at the conclusion of *First Capture* and thaw on ice.
  - Centrifuge the thawed RAH2 plate at 280 × g for 1 minute.

Procedure

1 Thoroughly vortex the Capture Target Buffer 3 tube until the solution is completely resuspended. Visually make sure that no crystal structures are present.

NOTE
If crystals and cloudiness are observed, vortex the Capture Target Buffer 3 tube until it is clear.

2 Remove the adhesive seal from the RAH2 plate.

3 Add the following reagents in the order listed to each well of the RAH2 plate:

Reagent	Volume (µl)
Resuspension Buffer	20
Capture Target Buffer 3	50
Coding Exome Oligos	5

4 Mix thoroughly as follows:
   a Seal the RAH2 plate with a Microseal 'B' adhesive seal. Make sure that the plate is tightly sealed to prevent potential evaporation. Use an adhesive seal roller to apply force to the seal and make sure that the seal is secured.
   b Shake the RAH2 plate on a microplate shaker at 1200 rpm for 1 minute

5 Centrifuge the RAH2 plate at 280 × g for 1 minute.

6 Place the sealed RAH2 plate on the pre-programmed thermal cycler. Close the lid, then select and run the **RNA HYB** program.
   a Choose the pre-heat lid option and set to 100°C
   b 95°C for 10 minutes
   c 18 cycles of 1 minute incubations, starting at 94°C, then decreasing 2°C per cycle
   d 58°C for forever

NOTE
Run the 58°C hybridization for 90 minutes. The RNA HYB program is set to 58°C for forever to make sure that the sample is at 58°C when the plate is removed.

7 Immediately remove the plate from the thermal cycler after 90 minutes of 58°C hybridization and proceed immediately to *Second Capture* on page 50.

NOTE
The total run time of the RNA HYB program is approximately 2 hours. Hybridizing longer than 2 hours results in a high degree of non-specific binding.

Second Capture

This process uses streptavidin beads to capture probes hybridized to the targeted regions of interest. Two heated wash procedures remove non-specific binding from the beads. The enriched library is then eluted from the beads and prepared for sequencing.

NOTE
These procedures are similar to the *First Capture* on page 43.

Consumables

Item	Quantity	Storage	Supplied By
2N NaOH (HP3)	1 tube	-25°C to -15°C	Illumina
Elute Target Buffer 2 (ET2)	1 tube	2°C to 8°C	Illumina
Enrichment Elution Buffer 1 (EE1)	1 tube	-25°C to -15°C	Illumina
Enrichment Wash Solution (EWS)	1 tube	-25°C to -15°C	Illumina
Streptavidin Magnetic Beads (SMB)	1 tube	2°C to 8°C	Illumina
1.7 ml microcentrifuge tube	1	15°C to 30°C	User
96-well MIDI plates	2	15°C to 30°C	User
Microseal 'B' adhesive seals	6	15°C to 30°C	User

Preparation

- Remove the 2N NaOH, Enrichment Elution Buffer 1, and Enrichment Wash Solution from -25°C to -15°C storage and thaw at room temperature.
- Remove the Elute Target Buffer 2 and Streptavidin Magnetic Beads from 2°C to 8°C storage and let stand at room temperature.
- Pre-heat the microheating system to 50°C.
- Label a new 96-well MIDI plate **RAW2** (RNA Access Wash 2) with a smudge resistant pen.

- Label a new 96-well MIDI plate **RAC1** (RNA Access Clean Up 1) with a smudge resistant pen.

Second Bind

1. Remove the RAH2 plate from the thermal cycler.
2. Centrifuge the room temperature RAH2 plate at 280 × g for 1 minute.
3. Remove the adhesive seal from the RAH2 plate. Take care when removing the seal to avoid spilling the contents of the wells.
4. Transfer the entire contents (~100 µl) from each well of the RAH2 plate to the corresponding well of the new 96-well MIDI plate labeled **RAW2**.
5. Vortex the Streptavidin Magnetic Beads tube until the beads are well dispersed, then add 250 µl well-mixed Streptavidin Magnetic Beads to the wells of the RAW2 plate.
6. Mix thoroughly as follows:
   a. Seal the RAW2 plate with a Microseal 'B' adhesive seal.
   b. Shake the RAW2 plate on a microplate shaker at 1200 rpm for 5 minutes
7. Let the RAW2 plate stand at room temperature for 25 minutes.
8. Centrifuge the RAW2 plate at 280 × g for 1 minute.
9. Remove the adhesive seal from the RAW2 plate.
10. Place the RAW2 plate on the magnetic stand for 2 minutes at room temperature or until the liquid is clear.
11. Carefully remove and discard all of the supernatant from each well of the RAW2 plate without disturbing the beads.
12. Remove the RAW2 plate from the magnetic stand.

Second Wash

1. Make sure that the Enrichment Wash Solution tube is at room temperature, then thoroughly vortex the tube.

   NOTE
   It is normal that the Enrichment Wash Solution can be cloudy after vortexing.

2. Add 200 µl Enrichment Wash Solution to each well of the RAW2 plate.

3 Mix thoroughly as follows:
   a Seal the RAW2 plate with a Microseal 'B' adhesive seal.
   b Shake the RAW2 plate on a microplate shaker at 1800 rpm for 4 minutes
   c Remove the adhesive seal from the RAW2 plate.
   d Gently pipette the entire volume of each well up and down to ensure complete resuspension of the sample.

4 Seal the RAW2 plate with a Microseal 'B' adhesive seal.

5 Incubate the RAW2 plate on the **pre-heated** microheating system, with the lid closed, at 50°C for 20 minutes.

6 Place the magnetic stand next to the microheating system for immediate access.

7 Remove the RAW2 plate from the microheating system and ***immediately*** place it on the magnetic stand for 2 minutes or until the liquid is clear.

8 Remove the adhesive seal from the RAW2 plate.

9 Immediately remove and discard all of the supernatant from each well of the RAW2 plate.

10 Remove the RAW2 plate from the magnetic stand.

11 Repeat steps 2–10 one time for a total of two Enrichment Wash Solution washes.

Second Elution

1 Add the following reagents in the order listed in a new 1.7 ml microcentrifuge tube to create the elution pre-mix. Multiply each volume by the number of pooled samples being prepared. The volumes include an excess amount for processing multiple samples.

Reagent	Volume (µl)
Enrichment Elution Buffer 1	28.5
2N NaOH	1.5
Total volume per enrichment	**30**

2 Vortex the elution pre-mix tube, then add **23** µl of the mix to each well of the RAW2 plate.

3 Mix thoroughly as follows:
   a Seal the RAW2 plate with a Microseal 'B' adhesive seal.
   b Shake the RAW2 plate on a microplate shaker at 1800 rpm for 2 minutes.

4 Let the RAW2 plate stand at room temperature for 2 minutes.

5 Centrifuge the RAW2 plate at 280 × g for 1 minute.

6 Carefully remove the adhesive seal from the RAW2 plate to avoid spilling the contents of the wells.

7 Place the RAW2 plate on the magnetic stand for 2 minutes or until the liquid is clear.

8 Transfer 21 µl of clear supernatant from each well of the RAW2 plate to the corresponding well of the new MIDI plate labeled **RAC1**. Take care not to disturb the beads.

NOTE
Illumina recommends using a 20 µl single channel or multichannel pipette set to 10.5 µl to perform two consecutive transfers of 10.5 µl. This technique reduces sample loss by making sure that all of the liquid is transferred without disturbing the beads.

9 Add 4 µl Elute Target Buffer 2 to each well of the RAC1 plate containing samples to neutralize the elution.

10 Mix thoroughly as follows:
   a Seal the RAC1 plate with a Microseal 'B' adhesive seal.
   b Shake the RAC1 plate on a microplate shaker at 1800 rpm for 1 minute.

11 Centrifuge the RAC1 plate at 280 × g for 1 minute.

12 Store the remaining reagents as follows:
   a Place the Elute Target Buffer 2 and Streptavidin Magnetic Beads tubes in 2°C to 8°C storage.
   b Place the 2N NaOH, Enrichment Elution Buffer 1, and Enrichment Wash Solution tubes in -25°C to -15°C storage.
   c Discard any remaining elution pre-mix.

Capture Sample Clean Up

This process uses AMPure XP beads to purify the captured library before PCR amplification.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
AMPure XP beads	45 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	3	15°C to 30°C	User

Preparation

- Remove the Resuspension Buffer and AMPure XP beads from 2°C to 8°C storage and bring them to room temperature.
- Label a new 96-well HSP plate **RAA** (RNA Access Amplification) with a smudge resistant pen.

Procedure

1. Remove the adhesive seal from the RAC1 plate.
2. Vortex the AMPure XP beads tube until the beads are well dispersed, then add 45 µl well-mixed AMPure XP beads to each well of the RAC1 plate.
3. Mix thoroughly as follows:
   a. Seal the RAC1 plate with a Microseal 'B' adhesive seal.
   b. Shake the RAC1 plate on a microplate shaker at 1800 rpm for 1 minute.
4. Incubate the RAC1 plate at room temperature for 5 minutes.
5. Centrifuge the RAC1 plate at 280 × g for 1 minute.

6 Remove the adhesive seal from the RAC1 plate.

7 Place the RAC1 plate on the magnetic stand for 2 minutes or until the liquid is clear.

8 Remove and discard all of the supernatant from each well of the RAC1 plate.

NOTE
Leave the RAC1 plate on the magnetic stand while performing the following 80% EtOH wash steps (9–12).

9 With the RAC1 plate on the magnetic stand, slowly add 200 µl freshly made 80% EtOH to each well without disturbing the beads.

10 Let the RAC1 plate stand at room temperature for 30 seconds.

11 Remove and discard the 80% EtOH from each well of the RAC1 plate.

12 Repeat steps 9–11 one time for a total of two 80% EtOH washes.

13 Using a 20 µl single channel or multichannel pipette, remove any remaining 80% EtOH from each well of the RAC1 plate without disturbing the beads.

14 Let the RAC1 plate stand at room temperature for 5 minutes to dry on the magnetic stand.

15 Remove the RAC1 plate from the magnetic stand.

16 Add 27.5 µl Resuspension Buffer to each well of the RAC1 plate. Do not touch the beads with the pipette tips.

17 Mix thoroughly as follows:
   a Seal the RAC1 plate with a Microseal 'B' adhesive seal.
   b Shake the RAC1 plate on a microplate shaker at 1800 rpm for 1 minute.

18 Incubate the RAC1 plate at room temperature for 2 minutes.

19 Centrifuge the RAC1 plate at 280 × g for 1 minute.

20 Remove the adhesive seal from the RAC1 plate.

21 Place the RAC1 plate on the magnetic stand for 2 minutes or until the liquid is clear.

22 Transfer 25 µl of clear supernatant from each well of the RAC1 plate to the corresponding well of the new HSP plate labeled **RAA**. Take care not to disturb the beads.

NOTE

Illumina recommends using a 20 µl single channel or multichannel pipette set to 12.5 µl to perform two consecutive transfers of 12.5 µl. This technique reduces sample loss by making sure that all of the liquid is transferred without disturbing the beads.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Second PCR Amplification* on page 57, you can safely stop the protocol here. If you are stopping, seal the RAA plate with a Microseal 'B' adhesive seal and store it at -25°C to -15°C for up to 7 days.

Second PCR Amplification

This process uses PCR to amplify the enriched DNA library for sequencing.

Consumables

Item	Quantity	Storage	Supplied By
Enhanced PCR Mix (EPM)	1 tube	-25°C to -15°C	Illumina
PCR Primer Cocktail (PPC)	1 tube	-25°C to -15°C	Illumina
Microseal 'A' film	1	15°C to 30°C	User
Microseal 'B' adhesive seal	1	15°C to 30°C	User

Preparation

- Remove the Enhanced PCR Mix and PCR Primer Cocktail from -25°C to -15°C storage and thaw on ice.
  - Briefly centrifuge the thawed Enhanced PCR Mix and PCR Primer Cocktail tubes for 5 seconds.

  NOTE
  If you do not intend to consume the Enhanced PCR Mix and PCR Primer Cocktail in one use, dispense the reagents into single use aliquots. Freeze the aliquots to avoid repeated freeze thaw cycles.

- Remove the RAA plate from -25°C to -15°C storage, if it was stored at the conclusion of *Second Capture* and thaw on ice.
  - Centrifuge the thawed RAA plate at 280 × g for 1 minute.
  - Remove the adhesive seal from the thawed RAA plate.

- Pre-program the thermal cycler with the following program and save as the **EPM AMP** program:
  - Choose the pre-heat lid option and set to 100°C
  - 98°C for 30 seconds
  - **10 cycles** of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 10°C

NOTE
Illumina has optimized the number of recommended PCR cycles for enrichment assays based on the level of pre-enrichment sample pooling and the size of the oligonucleotide set. Do not add or reduce the cycles of PCR, because it can compromise data quality.

Procedure

1. Add 5 µl PCR Primer Cocktail to each well of the RAA plate.
2. Add 20 µl Enhanced PCR Mix to each well of the RAA plate.
3. Mix thoroughly as follows:
   a. Seal the RAA plate with a Microseal 'A' film. Use an adhesive seal roller to apply force to the film and make sure that the film is secured.
   b. Shake the RAA plate on a microplate shaker at 1200 rpm for 1 minute
4. Centrifuge the RAA plate at 280 × g for 1 minute.
5. Place the sealed RAA plate on the pre-programmed thermal cycler. Close the lid, then select and run the **EPM AMP** program.
   a. Choose the pre-heat lid option and set to 100°C
   b. 98°C for 30 seconds
   c. **10 cycles** of:
      - 98°C for 10 seconds
      - 60°C for 30 seconds
      - 72°C for 30 seconds
   d. 72°C for 5 minutes
   e. Hold at 10°C

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Second PCR Clean Up* on page 60, the RAA plate can remain on the thermal cycler overnight. If you are stopping, replace the Microseal 'A' film with a Microseal 'B' adhesive seal and store the RAA plate at 2°C to 8°C for up to two days.

Second PCR Clean Up

This process uses AMPure XP beads to purify the enriched library and remove unwanted products.

Consumables

Item	Quantity	Storage	Supplied By
Resuspension Buffer (RSB)	1 tube	2°C to 8°C	Illumina
96-well HSP plate	1	15°C to 30°C	User
96-well MIDI plate	1	15°C to 30°C	User
AMPure XP beads	90 µl per sample	2°C to 8°C	User
Freshly prepared 80% ethanol (EtOH)	400 µl per sample	15°C to 30°C	User
Microseal 'B' adhesive seals	3	15°C to 30°C	User

Preparation

- Review Best Practices for *Handling Magnetic Beads*. See for information on how to access Best Practices on the Illumina website.
- Remove the Resuspension Buffer and AMPure XP beads from 2°C to 8°C storage and bring them to room temperature.
- Remove the RAA plate from 2°C to 8°C storage, if it was stored at the conclusion of *Second PCR Amplification* and let stand to bring to room temperature.
- Label a new 96-well MIDI plate **RAC2** (RNA Access Clean Up 2) with a smudge resistant pen.
- Label a new 96-well HSP plate **RAL** (RNA Access Library) with a smudge resistant pen.

Procedure

1. Centrifuge the RAA plate at 280 × g for 1 minute.
2. Remove the adhesive seal from the RAA plate.
3. Transfer the entire contents from each well of the RAA plate to the corresponding well of the new 96-well MIDI plate labeled **RAC2**.
4. Vortex the AMPure XP beads until the beads are well dispersed.
5. Add 90 µl well-mixed AMPure XP beads to each well of the RAC2 plate containing 50 µl of PCR amplified library.
6. Mix thoroughly as follows:
   a. Seal the RAC2 plate with a Microseal 'B' adhesive seal.
   b. Shake the RAC2 plate on a microplate shaker at 1800 rpm for 1 minute.
7. Incubate the RAC2 plate at room temperature for 5 minutes.
8. Centrifuge the RAC2 plate at 280 × g for 1 minute.
9. Remove the adhesive seal from the RAC2 plate.
10. Place the RAC2 plate on the magnetic stand at room temperature for 2 minutes or until the liquid is clear.
11. Carefully remove and discard all of the supernatant from each well of the RAC2 plate.

NOTE
Leave the RAC2 plate on the magnetic stand while performing the following 80% EtOH wash steps (12–15).

12. With the RAC2 plate on the magnetic stand, slowly add 200 µl freshly prepared 80% EtOH to each well without disturbing the beads.
13. Let the RAC2 plate stand at room temperature for 30 seconds.
14. Remove and discard the 80% EtOH from each well of the RAC2 plate.
15. Repeat steps 12–14 one time for a total of two 80% EtOH washes.
16. Using a 20 µl single channel or multichannel pipette, remove any remaining 80% EtOH from each well of the RAC2 plate without disturbing the beads.

17 Let the RAC2 plate stand at room temperature for 5 minutes to dry on the magnetic stand.

18 Remove the RAC2 plate from the magnetic stand.

19 Add 32 µl Resuspension Buffer to each well of the RAC2 plate. Do not touch the beads with the pipette tips.

20 Mix thoroughly as follows:
   a Seal the RAC2 plate with a Microseal 'B' adhesive seal.
   b Shake the RAC2 plate on a microplate shaker at 1800 rpm for 1 minute.

21 Incubate the RAC2 plate at room temperature for 2 minutes.

22 Centrifuge the RAC2 plate at 280 × g for 1 minute.

23 Remove the adhesive seal from the RAC2 plate.

24 Place the RAC2 plate on the magnetic stand for 2 minutes or until the liquid is clear.

25 Transfer 30 µl of clear supernatant from each well of the RAC2 plate to the corresponding well of the new HSP plate labeled **RAL**. Take care not to disturb the beads.

NOTE

Illumina recommends using a 20 µl single channel or multichannel pipette set to 15 µl to perform two consecutive transfers of 15 µl. This technique reduces sample loss by making sure that all of the liquid is transferred without disturbing the beads.

26 Seal the RAL plate with a Microseal 'B' adhesive seal.

SAFE STOPPING POINT

If you do not plan to proceed immediately to *Validate Library* on page 63, store the sealed RAL plate at -25°C to -15°C for up to 7 days. If the plate is stored for more than 7 days, requantify your library to guarantee the accuracy of your enrichment results.

Validate Library

Illumina recommends performing the following procedures for quality control analysis and quantification of your enriched library.

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 quantitation of DNA library templates. Quantify your libraries using qPCR according to the Illumina *Sequencing Library qPCR Quantification Guide (part # 11322363)*.

NOTE

See *Additional Resources* on page 7 for information on how to download the Illumina *Sequencing Library qPCR Quantification Guide (part # 11322363)* from the Illumina website.

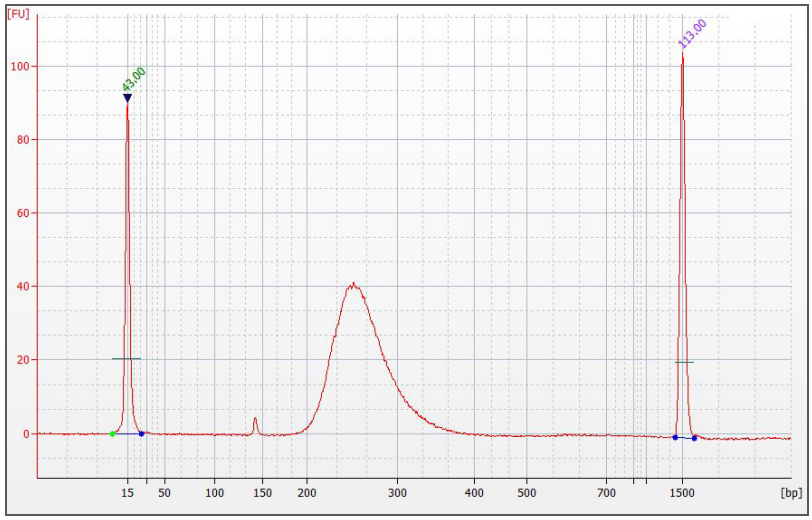
When quantitation is complete, proceed to cluster generation. For more information, see the cluster generation section of the user guide for your Illumina instrument.

[Optional] Assess Quality

Do the following to assess library quality:

1. Load 1 µl of the post-enriched library on one of the following:
   - Advanced Analytical Technologies Standard Sensitivity NGS Fragment Analysis Kit
   - Agilent High Sensitivity DNA Chip
2. Check the size of the library for a distribution of DNA fragments with a size range from approximately 200 bp–1 kb. Follow manufacturer instructions for either the Advanced Analytical Technologies Fragment Analyzer or Agilent Technologies 2100 Bioanalyzer, depending on the kit you are using.
   Depending on the level of indexing, insert size distribution can vary slightly, however the sample peak must not be significantly shifted compared to the example in Figure 9.

Figure 9 Example TruSeq RNA Access Library Prep Post-Enrichment Library Distribution



Supporting Information

Appendix A

Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed your kit contents, and obtained all of the requisite consumables and equipment.

Acronyms

Table 4 TruSeq RNA Access Library Prep Acronyms

Acronym	Definition
ALP	Adapter Ligation Plate
ATL	A-Tailing Mix
CAP	Clean Up ALP Plate
CCP	cDNA Clean Up Plate
cDNA	Complementary DNA
CEX	Coding Exome Oligos
CPP	Clean Up PCR Plate
CT3	Capture Target Buffer 3
DEPC	Diethylpyrocarbonate
DFP	Depleted RNA Fragmentation Plate
ds cDNA	Double-Stranded Complimentary DNA
dsDNA	Double-stranded DNA
DV	Fragment distribution value
EE1	Enrichment Elution Buffer 1
EPH	Elute, Prime, Fragment High Mix
EPM	Enhanced PCR Mix
ET2	Elute Target Buffer 2
EUC	Experienced User Card

Acronym	Definition
EWS	Enrichment Wash Solution
FFPE	Formalin-fixed, paraffin-embedded
FSA	First Strand Synthesis Act D Mix
HP3	2N NaOH
HSP	Hardshell Plate
IEM	Illumina Experiment Manager
LIG	Ligation Mix
LTF	Lab Tracking Form
PCR	Polymerase Chain Reaction
PMM	PCR Master Mix
PPC	PCR Primer Cocktail
RAA	RNA Access Amplification Plate
RAC1	RNA Access Clean Up Plate 1
RAC2	RNA Access Clean Up Plate 2
RAH1	RNA Access Hyb Plate 1
RAH2	RNA Access Hyb Plate 2
RAL	RNA Access Library Plate
RAW1	RNA Access Wash Plate 1
RAW2	RNA Access Wash Plate 2
RFU	Relative Fluorescence Unit
RIN	RNA integrity number

Acronym	Definition
rRNA	Ribosomal RNA
RSB	Resuspension Buffer
SMB	Streptavidin Magnetic Beads
SMM	Second Strand Marking Master Mix
STL	Stop Ligation Buffer
TSP	Target Sample Plate

Kit Contents

Check to make sure that you have all of the reagents identified in this section before starting the TruSeq RNA Access Library Prep protocol.

The TruSeq RNA Access Library Prep Kit is available in a Set A and a Set B. Each TruSeq RNA Access Library Prep Kit contains enough reagents to prepare up to 48 samples. When used together, sets A and B allow for pooling up to 24 samples using the 12 different indexes in each kit.

NOTE
The TruSeq RNA Access contains enough of each reagent for 4-plex pooling. Pooling at less than 4-plex results in the inability to process the number of samples supported by the kit.

Table 5 TruSeq RNA Access Kits

Kit Name	Catalog #	Number of Samples Supported	Number of Indexes
TruSeq RNA Access Library Prep Kit - Set A	RS-301-2001	48	12
TruSeq RNA Access Library Prep Kit - Set B	RS-301-2002	48	12

TruSeq RNA Access Library Prep Kit

The TruSeq RNA Access Library Prep Kit contains four boxes:

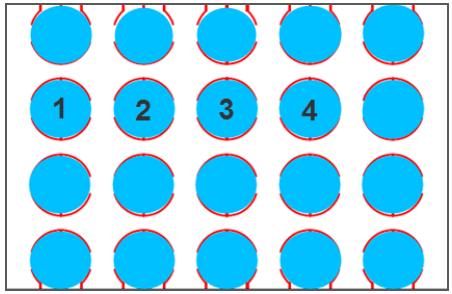
- cDNA Synthesis-PCR, Box 1
- 12 Index Set A or B, Box 2
- Capture Reagents, Box 3
- Coding Transcriptome, Box 4

cDNA Synthesis-PCR - Box 1

Store at -25°C to -15°C

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

Figure 10 TruSeq RNA Access, cDNA Synthesis-PCR 48 Samples, Box 1, part # 15052309



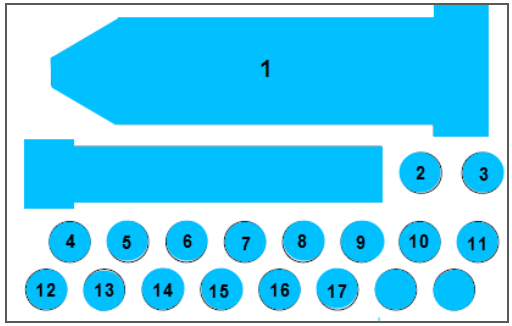
Slot	Reagent	Part #	Description
1	PMM	15026785	PCR Master Mix
2	PPC	15031748	PCR Primer Cocktail
3	FSA	15031094	First Strand Synthesis Act D Mix
4	SMM	15031098	Second Strand Marking Master Mix

12 Index Set - Box 2

You receive either a Set A or Set B, Box 2 in the kit, depending on the set ordered.

Store at -25°C to -15°C

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



Set A

TruSeq RNA Access, 12 Index Set A - 48 Samples, Box 2, part # 15052311

Slot	Reagent	Part #	Description
1	RSB	15026770	Resuspension Buffer
2	EPH	15029211	Elution Primer Fragmentation Mix
3	LIG	15026773	Ligation Mix
4	ATL	15012495	A-Tailing Mix
5	STL	15012546	Stop Ligation Buffer
6	AR013	15024655	RNA Adapter Index 13
7	AR014	15024656	RNA Adapter Index 14
8	AR015	15024657	RNA Adapter Index 15
9	AR016	15024658	RNA Adapter Index 16
10	AR018	15024660	RNA Adapter Index 18
11	AR019	15024661	RNA Adapter Index 19
12	AR002	15026634	RNA Adapter Index 2
13	AR004	15026636	RNA Adapter Index 4

Slot	Reagent	Part #	Description
14	AR005	15026637	RNA Adapter Index 5
15	AR006	15026638	RNA Adapter Index 6
16	AR007	15026640	RNA Adapter Index 7
17	AR012	15026645	RNA Adapter Index 12

Set B

TruSeq RNA Access, 12 Index Set B - 48 Samples, Box 2, part # 15052312

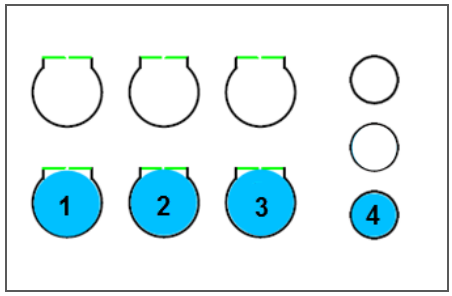
Slot	Reagent	Part #	Description
1	RSB	15026770	Resuspension Buffer
2	EPH	15029211	Elution Primer Fragmentation Mix
3	LIG	15026773	Ligation Mix
4	ATL	15012495	A-Tailing Mix
5	STL	15012546	Stop Ligation Buffer
6	AR020	15024662	RNA Adapter Index 20
7	AR021	15024663	RNA Adapter Index 21
8	AR022	15024664	RNA Adapter Index 22
9	AR023	15024665	RNA Adapter Index 23
10	AR025	15024667	RNA Adapter Index 25
11	AR027	15024668	RNA Adapter Index 27
12	AR001	15026633	RNA Adapter Index 1
13	AR003	15026635	RNA Adapter Index 3
14	AR008	15026641	RNA Adapter Index 8
15	AR009	15026642	RNA Adapter Index 9
16	AR010	15026643	RNA Adapter Index 10
17	AR011	15026644	RNA Adapter Index 11

Capture Reagents - Box 3

Store at 2°C to 8°C

This box is shipped on refrigerated gel packs. As soon as you receive your kit, store the components at 2°C to 8°C.

Figure 11 TruSeq RNA Access, Capture Reagents - 48 Samples, Box 3, part # 15052313



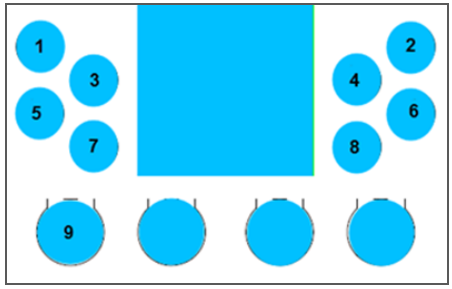
Slot	Reagent	Part #	Description
1–3	SMB	15015927	Streptavidin Magnetic Beads
2	ET2	15013008	Elute Target Buffer 2

Coding Transcriptome - Box 4

Store at -25°C to -15°C

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

Figure 12 TruSeq RNA Access, Coding Transcriptome-48 Samples, Box 4, part # 15052314



Slot	Reagent	Part #	Description
1–3	CEX	15034575	Coding Exome Oligos
4	CT3	15048799	Capture Target Buffer 3
5–6	EE1	15037034	Enrichment Elution Buffer 1
7	EPM	15041700	Enhanced PCR Mix
8	HP3	11324596	2N NaOH
9	EWS	15037119	Enrichment Wash Solution

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before starting the TruSeq RNA Access Library Prep protocol.

NOTE
The TruSeq RNA Access Library Prep protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Table 6 User-Supplied Consumables

Consumable	Supplier
1.5 ml RNase/DNase-free non-sticky tubes	Life Technologies, part # AM12450
1.7 ml microcentrifuge tubes	General lab supplier
10 µl barrier pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
20 µl barrier pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 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
1000 µl barrier pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier

Consumable	Supplier
96-well flat clear bottom black microplates Note: Used when quantifying samples with a SpectraMax M5 spectrofluorometer.	Corning, part # 3904
96-well storage plates, round well, 0.8 ml ("MIDI" plate)	Fisher Scientific, part # AB-0859
Adhesive seal roller	General lab supplier
Agencourt AMPure XP, 60 ml kit	Beckman Coulter, part # A63881/A63880
Aluminum foil	General lab supplier
Conical centrifuge tubes (15 ml or 50 ml)	General lab supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
Hard-Shell 96-well PCR Plates ("HSP" plate)	Bio-Rad, part # HSP-9601
Microseal 'A' film	Bio-Rad, part # MSA-5001
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
Nuclease-free ultra pure water	General lab supplier
One of the following (for library quality control): • Standard Sensitivity NGS Fragment Analysis Kit, 1–6000 bp (500 samples) • DNA 1000 Kit	• Advanced Analytical Technologies, part # DNF-473-0500 • Agilent Technologies, part # 5067-1504
RNase/DNase-free eight-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
RNaseZap (to decontaminate surfaces)	General lab supplier

Consumable	Supplier
SuperScript II Reverse Transcriptase	Invitrogen, part # 18064-014
Tris-HCl 10 mM, pH8.5	General lab supplier
Tween 20	Sigma, part # P7949
[Optional] 96-well 2 ml deep well plates (to aliquot reagents)	Thomson Instrument Company, part # 951652
[Optional] Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) Note: Used to concentrate a pooled library. Another option is to use a vacuum concentrator.	Millipore, part # UFC503008
[Optional] One of the following (for library quality assessment): • Standard Sensitivity NGS Fragment Analysis Kit, 1–6000 bp (500 samples) • High Sensitivity DNA Kit	• Advanced Analytical Technologies, part # DNF-473-0500 • Agilent Technologies, part # 4067-4626

Table 7 User-Supplied Equipment

Equipment	Supplier
DNA Engine Multi-Bay Thermal Cycler See *Thermal Cyclers* on page 81.	• Bio-Rad, part # PTC-0240G or • PTC-0220G, • with Alpha Unit, ALS-1296GC
Fluorometric quantitation with dsDNA binding dye reagents	General lab supplier

Equipment	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
High-Speed Microplate Shaker	VWR, catalog # • 13500-890 (110 V/120 V or • 14216-214 (230 V)
Magnetic stand-96	Life Technologies, part # AM10027
Microcentrifuge	General lab supplier
Microplate centrifuge	General lab supplier
MIDI plate insert for heating system Note: Two inserts are recommended to support successive heating procedures.	Illumina, catalog # BD-60-601

Equipment	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)
QuantiFluor dsDNA System or similar fluorometric-based DNA quantification system	Promega, catalog # E2670
SpectraMax M5 spectrofluorometer or similar fluorometric-based DNA quantification system	Molecular Devices, part # 0112-0159
Stroboscope	General lab supplier
Vortexer	General lab supplier
[Optional] Vacuum concentrator Note: Used to concentrate a pooled library. Another option is to use Amicon Ultra-0.5 centrifugal filter units.	General lab supplier

Thermal Cyclers

The following table lists the recommended settings for the Illumina recommended thermal cycler, as well as other comparable models. If your lab has a thermal cycler that is not listed, validate the thermal cycler before performing the TruSeq RNA Access Library Prep protocol.

Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad DNA Engine Tetrad 2	Calculated	Heated, Constant at 100°C	Polypropylene plates and tubes
MJ Research DNA Engine Tetrad	Calculated	Heated	Plate
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	Plate

TruSeq RNA Access Library Prep Kit Indexed Adapter Sequences

The TruSeq RNA Access Library Prep Kit contains the following indexed adapter sequences.

NOTE

- The index numbering is not contiguous. There is no Index 17, 24, or 26.
- The base in parentheses () indicates the base for the seventh cycle and is not considered as part of the index sequence. Record the index in the sample sheet as only six bases. For indexes 13 and above, the seventh base (in parentheses) might not be A, which is seen in the seventh cycle of the Index Read.
- For more information on the number of cycles used to sequence the Index Read, reference your instrument user guide.

Table 8 TruSeq RNA Access Library Prep Kit Set A Indexed Adapter Sequences

Adapter	Sequence	Adapter	Sequence
AR002	CGATGT(A)	AR013	AGTCAA(C)
AR004	TGACCA(A)	AR014	AGTTCC(G)
AR005	ACAGTG(A)	AR015	ATGTCA(G)
AR006	GCCAAT(A)	AR016	CCGTCC(C)
AR007	CAGATC(A)	AR018	GTCCGC(A)
AR012	CTTGTA(A)	AR019	GTGAAA(C)

Table 9 TruSeq RNA Access Library Prep Kit Set B Indexed Adapter Sequences

Adapter	Sequence	Adapter	Sequence
AR001	ATCACG(A)	AR020	GTGGCC(T)
AR003	TTAGGC(A)	AR021	GTTTCG(G)
AR008	ACTTGA(A)	AR022	CGTACG(T)
AR009	GATCAG(A)	AR023	GAGTGG(A)
AR010	TAGCTT(A)	AR025	ACTGAT(A)
AR011	GGCTAC(A)	AR027	ATTCCT(T)

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 10 Illumina General Contact Information

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

Table 11 Illumina Customer Support Telephone Numbers

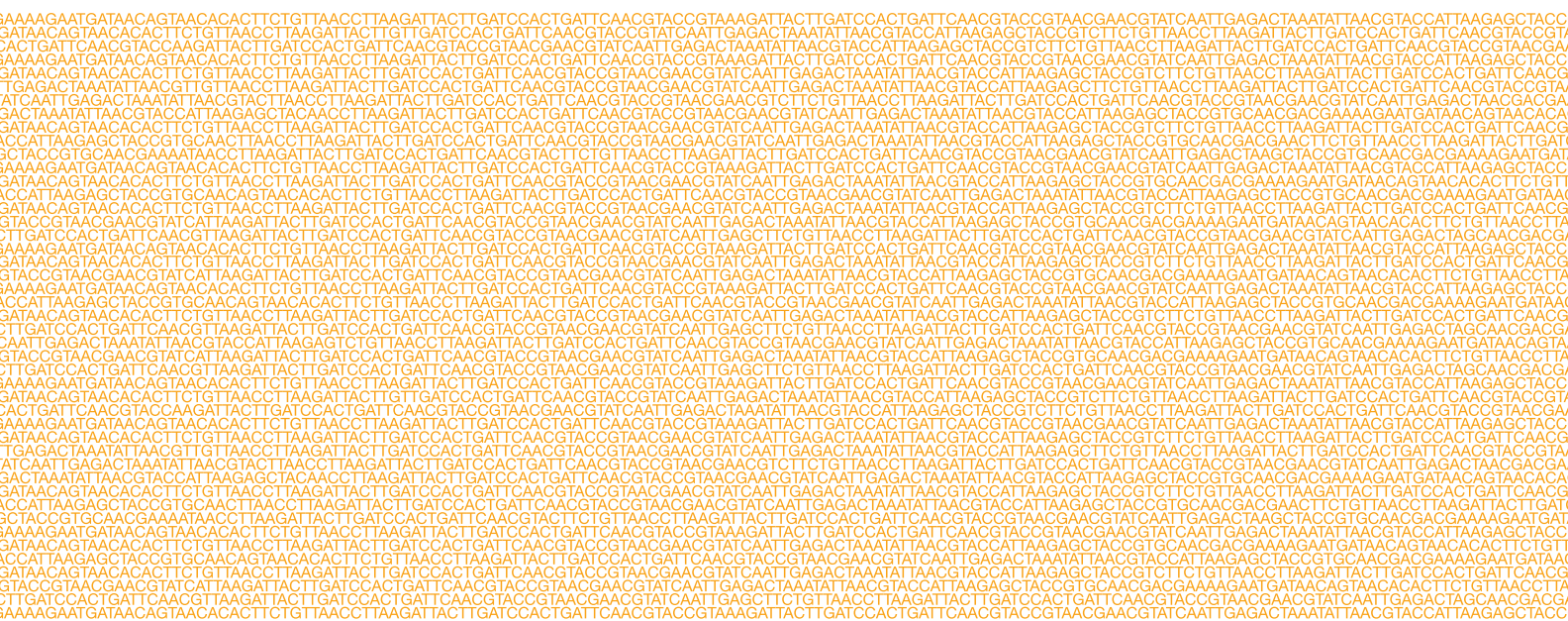
Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994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

Safety Data Sheets

Safety data sheets (SDSs) are available on the Illumina website at support.illumina.com/sds.ilmn.

Product Documentation

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



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