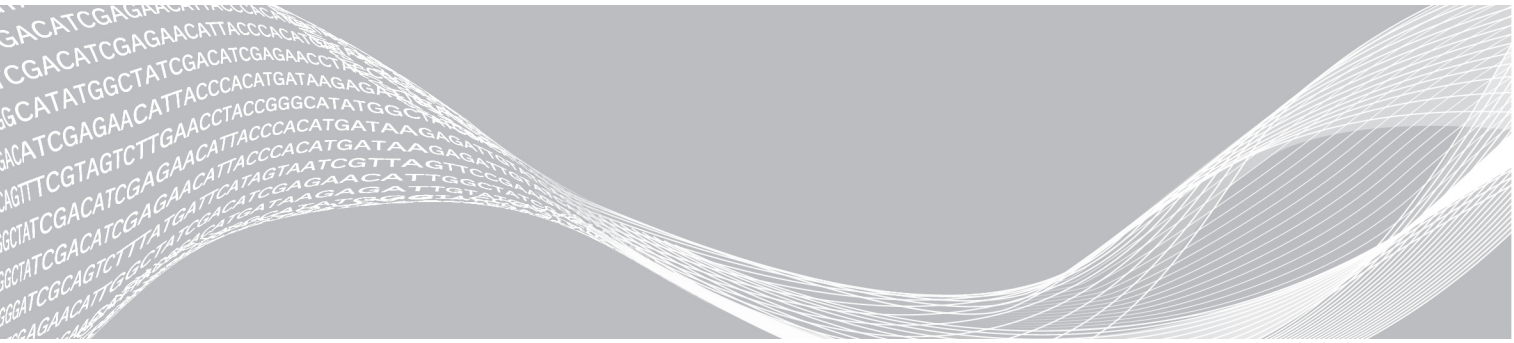
illumina®

TruSeq RNA Exome

Reference Guide



Document # 1000000039582 v01

ILLUMINA PROPRIETARY

March 2018

Revision History

Document	Date	Description of Change
1000000039582 v01	March 2018	• Changed Prepare for Pooling heading to Prepare Adapter Setup • Add Index Adapters procedure, removed the "Centrifuge STL at 600 x g for 5 seconds." step. • Enrich DNA Fragments|Consumables, added 96-well midi plate • Amplify DNA Fragment changed shake time to 20 seconds. • Hybridize Probes| Preparation, changed "58C for 90 minutes" to "58C for forever for 90 minutes." • Clean UP Amplified DNA, removed "Centrifuge at 280 x g for 1 minute.", added "Transfer contents from PCR plate to the CPP plate.", Incubate step 4 set to 5 minutes, Air-dry step set to 5 minutes. • Check Library Quality, corrected graphics.
1000000039582 v00	October 2017	Initial release.

Table of Contents

Chapter 1 Overview

Introduction

This protocol explains how to convert total RNA into a library of known strand origin, and then capture the coding regions of the transcriptome using reagents for the Illumina® TruSeq® RNA Exome workflow. The library is suitable for subsequent cluster generation and DNA sequencing.

The RNA is fragmented using divalent cations under elevated temperature. cDNA is generated from the cleaved RNA fragments using random priming during first and second strand synthesis. Then, sequencing adapters are ligated to the resulting double-stranded cDNA fragments. The coding regions of the transcriptome are 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
- Single-plex enrichment for up to 48 samples
- Strand information on RNA transcripts
- High throughput automation-friendly procedures

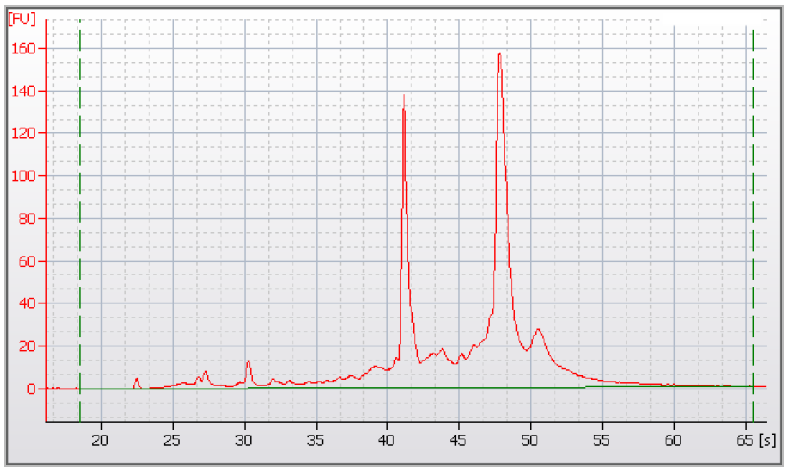
RNA Input Recommendations

Total RNA Input

- The protocol is optimized for 10–100 ng of human total RNA.
  - Lower amounts might result in low yield and inefficient ligation.
- 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 lower quality RNA might require further optimization to determine the input amount.

- 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 Universal Human Reference (UHR) starting RNA Bioanalyzer trace.

Figure 1 Starting RNA Bioanalyzer Trace



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

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, use 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.
  - Use NanoDrop to determine FFPE RNA concentration.
  - For samples that border a quality classification, error towards the higher end of the input recommendation.

NOTE

For more information on calculating the DV_{200}, see the *Evaluating RNA Quality from FFPE Samples* tech note for the TruSeq RNA Exome workflow. See *Additional Resources* on page 1 for information on how to download the tech note from the Illumina website.

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

- The following are examples of high, medium, and low quality FFPE traces, plus a trace of FFPE quality that is not recommended for use with TruSeq RNA Exome workflow.

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

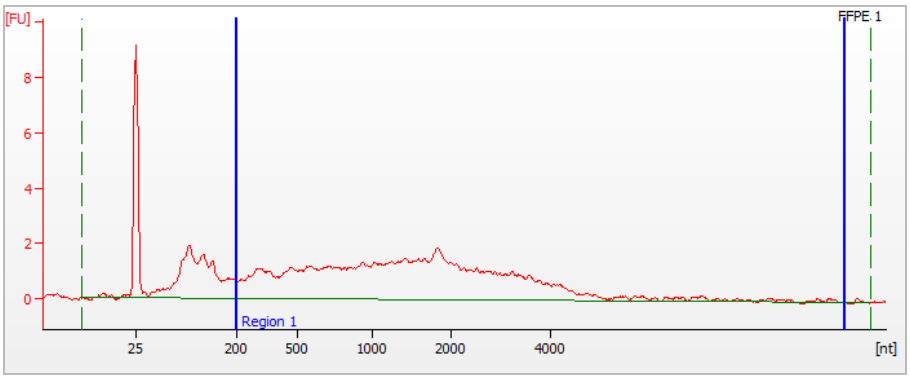


Figure 3 Example: Medium Quality FFPE (DV_{200} = 55%)

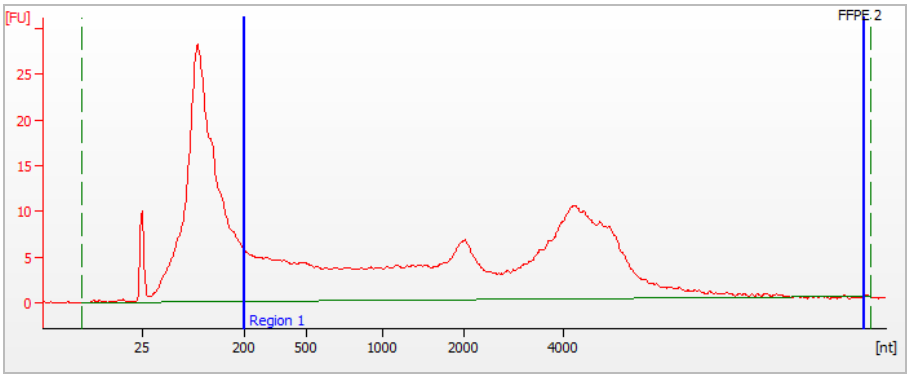


Figure 4 Example: Low Quality FFPE (DV_{200} = 30%)

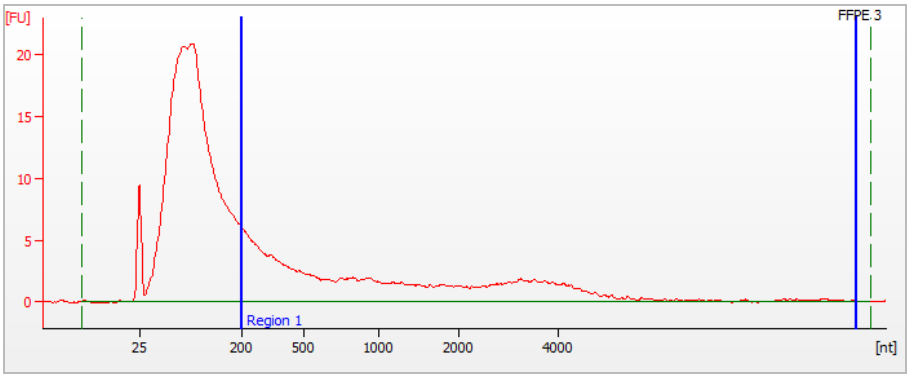
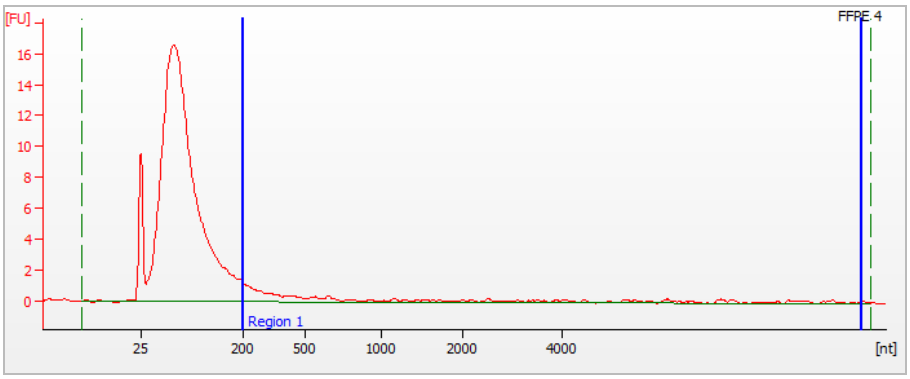


Figure 5 Example: FFPE Quality Not Recommended for Use (DV_{200} = 8%)



Positive Control

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	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 RNA Exome Checklist (document # 1000000040889)	Provides a checklist of the protocol steps. The checklist is intended for experienced users.
Evaluating RNA Quality from FFPE Samples tech note	Provides effectivity profiles for FFPE RNA.
TruSeq Library Prep Pooling Guide (document # 15042173)	Provides TruSeq pooling guidelines for preparing libraries for Illumina sequencing systems that require balanced index combinations. Review this guide before beginning library preparation.
Illumina Adapter Sequences (document # 1000000002694)	Provides the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.
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)	Provide information about creating and editing appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate.
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 Sequence Hub sequencing data analysis tool that also enables you to organize samples, libraries, pools, and sequencing runs in a single environment.
Local Run Manager Software Guide (document # 100000002701)	Provides an overview of the Local Run Manager (LRM) software, instructions for using software features, and instructions for installing analysis modules on the instrument computer.

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

Chapter 2 Protocol

Introduction

This section describes the TruSeq RNA Exome workflow protocol.

- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- Review Best Practices before proceeding. See *Additional Resources* on page 1 for information on how to access TruSeq RNA Exome workflow Best Practices on the Illumina website.
- Before proceeding, confirm kit contents and make sure that you have the required equipment and consumables. For more information, see *Supporting Information* on page 32.

Tips and Techniques

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

Avoiding Cross-Contamination

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

Sealing the Plate

- Always seal the 96-well plate before the following steps in the protocol:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps
- Apply the adhesive seal to cover the plate, and seal with a rubber roller.

- Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' for shaking, centrifuging, and long-term storage.
- Microseal 'A' adhesive film is used for thermal cycling steps to prevent evaporation.

Plate Transfers

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

Centrifugation

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

Handling Beads

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

Library Prep Workflow

Figure 6 TruSeq RNA Exome workflow

Prepare Adapter Setup

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.

Include a common index in each column. A common index facilitates pipetting operations when dispensing index adapters and pooling indexed libraries.

Review the planning steps in the *TruSeq Library Prep Pooling Guide (document # 15042173)* when preparing libraries for Illumina sequencing systems that require balanced index combinations.

Arrange 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, see *Total RNA Input* on page 1 for more information. Add the EPH to the sample, but do not perform the incubation steps 5–6 in this procedure.

Consumables

- EPH (Elute, Prime, Fragment High Mix)
- RSB (Resuspension Buffer)
- Total RNA (10 ng fresh/frozen RNA per reaction or 20–100 ng FFPE RNA per reaction)
- Ultrapure Water
- 96-well Hard-Shell 0.3 ml PCR plate
- Microseal 'B' adhesive seal

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EPH	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
RSB	-25°C to -15°C	Thaw at room temperature. Store at 2°C to 8°C after the initial thaw.

2 Save the following Elution 2-Frag-Prime program on the thermal cycler.
   - Choose the preheat lid option and set to 100°C
   - 94°C for 8 minutes
   - Hold at 4°C
   - Each well contains 17 µl
3 Set the centrifuge to 15°C to 25°C.
4 Label a new Hard-Shell PCR plate DFP with a marker.

Procedure

1 Dilute the total RNA in nuclease-free ultrapure water to a final volume of 8.5 µl in each well of the DFP plate.
2 Add 8.5 µl EPH to each well.
3 Apply the seal and shake at 1600 rpm for 20 seconds.
4 Apply the seal and centrifuge at 280 × g for 1 minute.

WARNING

If starting with FFPE RNA, do not perform the incubation procedure in steps 5–6. Proceed immediately to *Synthesize First Strand cDNA* on page 10.

5 Place on the preprogrammed thermal cycler and run the Elution 2-Frag-Prime program.
6 Remove from thermal cycler when it reaches 4° C and 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
- 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 38.

Preparation

1 Prepare the following consumables.

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

2 Save the following Synthesize 1st Strand program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - 25°C for 10 minutes
   - 42°C for 15 minutes
   - 70°C for 15 minutes
   - Hold at 4°C
   - Each well contains 25 µl

3 Make sure that the microplate shaker is properly calibrated to 1000 rpm using a stroboscope.

NOTE

The FSA with SuperScript II added is stable to additional freeze-thaw cycles and can be used for subsequent experiments. If more than 6 freeze-thaw cycles are anticipated, divide the FSA and SuperScript II mix into smaller aliquots and store at -25°C to -15°C.

Procedure

1 Centrifuge FSA at 600 × g for 5 seconds.

2 Add 50 µl SuperScript II to FSA. Pipette to mix. Then apply the seal and 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 SuperScript II and FSA mixture to each well.

4 Apply the seal and shake at 1600 rpm for 20 seconds.

5 Place on the preprogrammed thermal cycler and run the Synthesize 1st Strand program.

6 When the thermal cycler reaches 4°C, remove the DFP plate from the thermal cycler.

Synthesize Second Strand cDNA

This process displaces the RNA template, synthesizes a replacement strand, and incorporates uridine in place of dTTP to generate ds cDNA. The incorporation of uridine prevents the second strand synthesis 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
- Freshly prepared 80% ethanol (EtOH)
- 96-well midi plates (2)
- Microseal 'B' adhesive seals

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
SMM	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
AMPure XP beads	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

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

3 Preheat the thermal cycler to 16°C.

4 Label a new Hard-Shell PCR plate ALP with a marker.

5 Label a new midi plate CCP with a marker.

Procedure

Add SMM

1 Add 5 µl RSB to each well.

2 Centrifuge SMM at 600 × g for 5 seconds.

3 Add 20 µl SMM to each well.

4 Apply the seal and shake at 1600 rpm for 20 seconds.

5 Apply the seal and centrifuge at 280 × g for 1 minute.

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

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

Purify cDNA

1 Add 90 µl AMPure XP beads to the CCP plate.

2 Transfer all from the DFP plate to the corresponding well of the CCP plate.

3 Apply the seal and shake at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 5 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 Leave the CCP plate on the magnetic stand when performing the following wash step.

9 Wash 2 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.

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

11 Air-dry on the magnetic stand for 5 minutes.

12 Remove from the magnetic stand.

13 Add 17.5 µl RSB to each well.

14 Apply the seal and shake at 1800 rpm for 2 minutes.

15 Incubate at room temperature for 2 minutes.

16 Centrifuge at 280 × g for 1 minute.

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

18 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 seven days.

Adenylate 3′ Ends

A single 'A' nucleotide is added to the 3′ ends of the blunt fragments to prevent them from ligating to 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 and concatenated adapters) formation.

Consumables

- ATL (A-Tailing Mix)
- RSB (Resuspension Buffer)
- Microseal 'B' adhesive seals
- Bucket with ice

Preparation

1 Prepare the following consumables.

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

2 Preheat 2 microheating systems, one to 37°C and another to 70°C.

Procedure

1 Centrifuge ATL at 600 × g for 5 seconds.

2 Add 2.5 µl RSB to each well.

3 Add 12.5 µl ATL to each well.

4 Apply the seal and shake at 1800 rpm for 2 minutes.

5 Centrifuge at 280 × g for 1 minute.

6 Place on the 37°C microheating system with the lid closed for 30 minutes.

7 Move to the 70°C microheating system with the lid closed for 5 minutes.

8 Place on ice for 1 minute.

Ligate Adapters

This process ligates indexing adapters to the ends of the ds cDNA fragments, which prepares them for enrichment by PCR and sequencing.

Index adapters must be ordered separately from the Library Prep components. For information on compatible index adapters, see *Supporting Information* on page 32.

Consumables

- LIG (Ligation Mix)
- RNA Adapters
- RSB (Resuspension Buffer)
- AMPure XP beads
- STL (Stop Ligation Buffer)
- Freshly prepared 80% ethanol (EtOH)
- 96-well midi plate
- 96-well Hard-Shell 0.3 ml PCR plate
- Microseal 'B' adhesive seals

About Reagents

- 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
RNA Adapters	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
STL	-25°C to -15°C	Thaw at room temperature. Return to storage after use.

Item	Storage	Instructions
RSB	2°C to 8°C	Bring to room temperature.
AMPure XP beads	2°C to 8°C	Let stand at least 30 minutes to bring to room temperature.

2 Preheat a microheating system to 30°C.

3 Label a new Hard-Shell PCR plate PCR with a marker.

4 Label a new midi plate CAP with a marker.

Procedure

Add Index Adapters

1 Centrifuge the RNA Adapter tubes at 600 × g for 5 seconds.

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

3 Add the following reagents in the order listed to each well.
- RSB (2.5 µl)
- LIG (2.5 µl)
- RNA adapters (2.5 µl)

4 Apply the seal and shake at 1800 rpm for 2 minutes.

5 Centrifuge at 280 × g for 1 minute.

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

7 Add 5 µl STL to each well.

8 Apply the seal and shake at 1800 rpm for 2 minutes.

9 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 Apply the seal and shake at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 5 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 2 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 5 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 Apply the seal and shake at 1800 rpm for 2 minutes.

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 seven days.

Enrich DNA Fragments

This process uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the 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
- Freshly prepared 80% ethanol (EtOH)
- 96-well Hard-Shell 0.3 ml PCR plate
- 96-well midi plate
- Microseal 'A' film
- Microseal 'B' adhesive seals

NOTE

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

About Reagents

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

Preparation

1 Prepare the following consumables.

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

2 Save the following 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 or 10 seconds
     - 60°C for 30 seconds
     - 72°C for 30 seconds
   - 72°C for 5 minutes
   - Hold at 4°C
   - Each well contains 50 µl
3 Label a new midi plate PPP with a marker.
4 Label a new Hard-Shell PCR plate TSP1 with a marker.

Procedure

Amplify DNA Fragments

1 Place on ice and add 5 µl PPC to each well.
2 Add 25 µl PMM to each well.
3 Apply the seal and shake at 1600 rpm for 20 seconds.
4 Apply the seal and centrifuge at 280 × g for 1 minute.

NOTE

If you have separate pre-PCR and post-PCR areas, move to the post-PCR area.

5 Place on the preprogrammed thermal cycler and run the PCR program.

Clean Up Amplified DNA

1 Add 50 µl AMPure XP beads to each well.
2 Transfer contents from PCR plate to the CPP plate.
3 Apply the seal and shake at 1800 rpm for 2 minutes.
4 Incubate at room temperature for 5 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 2 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 5 minutes.
11 Remove from the magnetic stand.
12 Add 17.5 µl RSB to each well.
13 Apply the seal and shake at 1800 rpm for 2 minutes.
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 15 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 seven days.

Check Libraries

Quantify Library

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

Check Library Quality

1 If using a Standard Sensitivity NGS Fragment Analysis Kit on an Advanced Analytical Fragment Analyzer:
   a Dilute the DNA library 1:1 with RSB.
   b Run 1 µl diluted DNA library.
2 If using a DNA 1000 chip on an Agilent Technologies 2100 Bioanalyzer, run 1 µl undiluted DNA library.
3 Check the size and purity of the sample. Expect the final product to be a band at ~260 bp.

Figure 7 Example Library Size Distribution

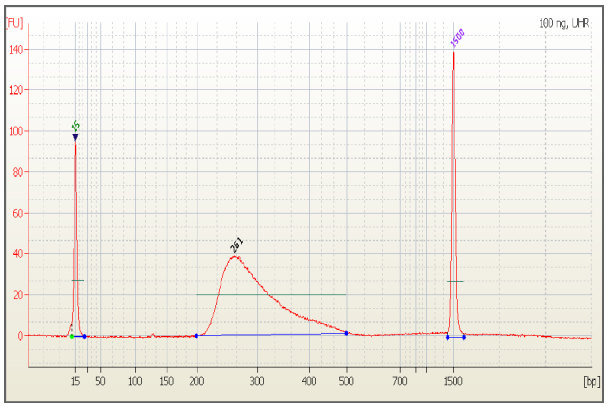
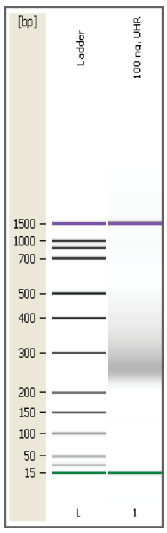


Figure 8 TruSeq RNA Exome workflow 260 bp PCR Product



First Hybridization of Probes

This step combines DNA libraries containing unique indexes into a single pool, and then binds targeted regions of the DNA with capture probes.

Consumables

- CT3 (Capture Target Buffer 3)
- CEX (Coding Exome Oligos)
- RSB (Resuspension Buffer)
- 96-well Hard-Shell 0.3 ml PCR plate
- Microseal 'B' adhesive seal
- **[Optional]** Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) (1 per pooled sample)

About Reagents

- Before using CT3, vortex to resuspend the solution. Make sure that no crystal structures are present. If crystals and cloudiness are observed, vortex until the solution is clear.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
CEX	-25°C to -15°C	Thaw at room temperature.
CT3	-25°C to -15°C	Thaw at room temperature.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Save the RNA HYB program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 95°C for 10 minutes
- 18 cycles of 1 minute each, starting at 94°C, then decreasing 2°C per cycle
- 58°C for forever for 90 minutes

NOTE

Hybridizing longer than the programmed 2 hours results in a high degree of nonspecific binding.

3 Label a new Hard-Shell PCR plate REH1 (RNA Exome Hyb 1) with a marker.

Pool Libraries

Combine 200 ng of each DNA library for pooling.

NOTE

The TruSeq RNA Exome workflow contains enough of each reagent for up to 1 to 4-plex pooling.

For best results, pool only 4 or fewer libraries.

Pooling an odd number of libraries does not affect data. For example, for 5 libraries, you can pool 2 libraries and 3 libraries or 4 libraries and 1 library.

Table 2 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 > 45 µl, use a vacuum concentrator or Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa) to concentrate the pooled sample to 45 µl.
  - If you are using a vacuum concentrator, use a no heat setting and a medium drying rate.

- If you are using an Amicon Ultra-0.5 centrifugal filter unit (0.5 ml, 30 kDa), it is not required to 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 total volume is < 45 µl, increase the volume to 45 µl with RSB.

Procedure

1. Add the following items in the order listed to each well of the REH1 plate.
   - DNA library sample or pool (45 µl)
   - CT3 (50 µl)
   - CEX (5 µl)
2. For single-plex, add the following items in the order listed to each well of the REH1 plate:
   - DNA library sample 200ng (11.25 µl)
   - CT3 (12.5 µl)
   - CEX (1.25 µl)
3. Shake at 1200 rpm for 1 minute.
4. Centrifuge at 280 × g for 1 minute.
5. Place on the thermal cycler and run the RNA HYB program.
   - For 2-4 plex pools, each well contains 100 µl.
   - For single-plex pools, each well contains 25 µl.
6. Remove from the thermal cycler immediately after the 90-minute incubation.

First Capture of Hybridized Probes

This process mixes the DNA library with capture probes to targeted regions of interest. Next, Streptavidin Magnetic Beads (SMB) are used to capture hybridized probes. Two heated washes remove nonspecific binding from the beads. The enriched library is then eluted from the beads and prepared for a second round of hybridization.

Consumables

- EE1 (Enrichment Elution Buffer 1)
- ET2 (Elute Target Buffer 2)
- EWS (Enrichment Wash Solution)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- 96-well Hard-Shell 0.3 ml PCR plate
- 96-well midi plate
- 1.7 ml microcentrifuge tube
- Microseal 'B' adhesive seals

About Reagents

- EWS can be cloudy after reaching room temperature.
- Vortex EWS before use.

- Make sure that you use SMB (2 ml tube) and not SPB (15 ml tube) for this procedure.
- Invert and vortex SMB to mix before use.
- Discard elution premix after use.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EE1	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
EWS	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
HP3	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
ET2	2°C to 8°C	Let stand at room temperature. Return to storage after use.
SMB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Return to storage after use.

2 Preheat a microheating system with midi plate insert to 50°C.

3 Label plates with a marker as follows.
- REW1 - midi
- REH2 - Hard-Shell PCR

Procedure

First Bind

1 Centrifuge at 280 × g for 1 minute.

2 Transfer all (~100 µl for pooled libraries, ~25 µl for 1-plex) to the corresponding well of the REW1 plate.

NOTE

If you see a greater than 15% sample loss, do not proceed with the protocol. Poor sealing or insufficient heating of the lid can cause sample loss.

3 Add 250 µl (62.5 µl for 1-plex) SMB to each well.

4 Shake at 1200 rpm for 5 minutes.

5 Incubate at room temperature for 25 minutes.

6 Centrifuge at 280 × g for 1 minute.

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

8 Remove and discard all supernatant from each well.

9 Remove from the magnetic stand.

First Wash

1 Wash two times as follows.

   a Add 200 µl (50 µl for 1-plex) EWS to each well.
   b Seal the midi plate and shake at 1800 rpm for 4 minutes.
   c Remove seal and pipette to completely resuspend the bead pellet further.
   d Seal and place the midi plate on the 50°C microheating system with the lid closed for 20 minutes.
   e Remove seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).
   f Remove and discard all supernatant from each well.
   g Remove the midi plate from the magnetic stand.

First Elution

1 Create elution premix in a 1.7 ml microcentrifuge tube, and then vortex to mix.
   - EE1 (28.5 µl)
   - HP3 (1.5 µl)

2 For 1-plex, create elution premix in a 1.7 microcentrifuge tube, and then vortex to mix.
   - EE1 (9.5 µl)
   - HP3 (0.5 µl)

3 Add 23 µl (10 µl for 1-plex) elution premix to each well.

4 Shake at 1800 rpm for 2 minutes.

5 Incubate at room temperature for 2 minutes.

6 Centrifuge at 280 × g for 1 minute.

7 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

8 Transfer 21 µl (9 µl for 1-plex) supernatant to the corresponding well of the REH2 plate.

9 Add 4 µl (1.7 µl for 1-plex) ET2 to each well.

10 Shake at 1200 rpm for 1 minute.

11 Centrifuge at 280 × g for 1 minute.

SAFE STOPPING POINT

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

Second Hybridization of Probes

This step binds targeted regions of the enriched DNA with capture probes a second time. This second hybridization ensures high specificity of the captured regions.

Consumables

- CT3 (Capture Target Buffer 3)
- CEX (Coding Exome Oligos)
- RSB (Resuspension Buffer)
- Microseal 'B' adhesive seals

About Reagents

- Before using CT3, vortex to resuspend the solution. Make sure that no crystal structures are present. If crystals and cloudiness are observed, vortex until the solution is clear.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
CEX	-25°C to -15°C	Thaw at room temperature.
CT3	-25°C to -15°C	Thaw at room temperature.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

Procedure

1 Add the following reagents in the order listed to each well that contains a sample.
- RSB (20 µl)
- CT3 (50 µl)
- CEX (5 µl)

2 For 1-plex, add the following reagents in the order listed to each well that contains a sample.
- RSB (0.55 µl)
- CTE (12.5 µl)
- CEX (1.25 µl)

3 Shake at 1200 rpm for 1 minute.

4 Centrifuge at 280 × g for 1 minute.

5 Place on the preprogrammed thermal cycler and run the RNA HYB program.
- For 2-4 plex pools, each well contains 100 µl.
- For single-plex pools, each well contains 25 µl.

6 Remove from the thermal cycler immediately after the 90-minute incubation.

Second Capture of Hybridized Probes

This step uses SMB (Streptavidin Magnetic Beads) to capture probes hybridized to the targeted regions of interest. Two heated washes remove nonspecific binding from the beads. The enriched library is then eluted from the beads and prepared for sequencing.

Consumables

- EE1 (Enrichment Elution Buffer 1)
- ET2 (Elute Target Buffer 2)
- EWS (Enrichment Wash Solution)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- 96-well midi plates (2)
- 1.7 ml microcentrifuge tube

- Microseal 'B' adhesive seals

About Reagents

- EWS can be cloudy after reaching room temperature.
- Vortex EWS before use.
- Invert SMB to mix before use.
- Discard elution premix after use.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EE1	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
EWS	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
HP3	-25°C to -15°C	Thaw at room temperature. Return to storage after use.
ET2	2°C to 8°C	Let stand at room temperature. Return to storage after use.
SMB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Return to storage after use.

2 Preheat a microheating system with midi plate insert to 50°C.

3 Label plates with a marker as follows.
- REW1 - midi
- REH2 - Hard-Shell PCR

Procedure

Second Bind

1 Centrifuge at 280 × g for 1 minute.

2 Transfer all (~100 µl; ~25 µl for 1-plex) supernatant to the corresponding well of the REW2 plate.

NOTE

If you see a greater than 15% sample loss, do not proceed with the protocol. Poor sealing or insufficient heating of the lid can cause sample loss.

3 Add 250 µl (62.5 µl for 1-plex) SMB to each well.

4 Shake at 1200 rpm for 5 minutes.

5 Incubate at room temperature for 25 minutes.

6 Centrifuge at 280 × g for 1 minute.

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

8 Remove and discard all supernatant from each well.

9 Remove from the magnetic stand.

Second Wash

1 Wash two times as follows.

   a Add 200 µl (50 µl for 1-plex) EWS to each well.
   b Seal the midi plate and shake at 1800 rpm for 4 minutes.
   c Remove the seal and pipette to completely resuspend the bead pellet further.
   d Seal and place the midi plate on the 50°C microheating system with the lid closed for 20 minutes.
   e Remove seal and place the midi plate on a magnetic stand and wait until the liquid is clear (~2 minutes).
   f Remove and discard all supernatant from each well.
   g Remove the midi plate from the magnetic stand.

Second Elution

1 Create elution premix in a 1.7 ml microcentrifuge tube, and then vortex to mix.
   - EE1 (28.5 µl)
   - HP3 (1.5 µl)

2 For 1-plex, create elution premix in a 1.7 ml microcentrifuge tube, and then vortex to mix.
   - EE1 (9.5 µl)
   - HP3 (0.5 µl)

3 Add 23 µl (10 µl for 1-plex) elution premix to each well.

4 Shake at 1800 rpm for 2 minutes.

5 Incubate at room temperature for 2 minutes.

6 Centrifuge at 280 × g for 1 minute.

7 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

8 Transfer 21 µl (9 µl for 1-plex) supernatant to the corresponding well of the REC1 plate.

9 Add 4 µl (1 µl for 1-plex) ET2 to each well.

10 Shake at 1800 rpm for 1 minute.

11 Centrifuge at 280 × g for 1 minute.

Clean Up Captured Library

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

Consumables

- RSB (Resuspension Buffer)
- AMPure XP beads
- Freshly prepared 80% ethanol (EtOH)
- 96-well Hard-Shell 0.3 ml PCR plate
- Microseal 'B' adhesive seals

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
RSB	2°C to 8°C	Bring to room temperature.
AMPure XP beads	2°C to 8°C	Bring to room temperature.

2 Prepare fresh 80% ethanol from absolute ethanol.

3 Label a new Hard-Shell PCR plate RAA with a marker.

Procedure

1 Add 45 µl (18 µl for 1-plex) AMPure XP beads to each well.

2 Apply the seal and shake at 1800 rpm for 1 minute.

3 Incubate at room temperature for 5 minutes.

4 Centrifuge at 280 × g for 1 minute.

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

6 Remove and discard all supernatant from each well.

7 Wash 2 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.

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

9 Air-dry on the magnetic stand for 5 minutes.

10 Remove from the magnetic stand.

11 Add 27.5 µl RSB to each well.

12 Apply the seal and shake at 1800 rpm for 1 minute.

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 minutes).

16 Transfer 25 µl supernatant to the corresponding well of the REA plate.

SAFE STOPPING POINT

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

Amplify Enriched Library

This step uses a 10-cycle PCR program to amplify the enriched library.

Consumables

- EPM (Enhanced PCR Mix)
- PPC (PCR Primer Cocktail)
- Microseal 'A' film
- Microseal 'B' adhesive seal

NOTE

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

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice.
PPC	-25°C to -15°C	Thaw at room temperature.

2 Save the following EPM AMP program on the thermal cycler:
- Choose the preheat 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
- Each well contains 50 µl

Procedure

1 Remove seal and add 5 µl PPC to each well.

2 Add 20 µl EPM to each well.
The total volume per well is 50 µl.

3 Apply the seal and shake at 1200 rpm for 1 minute.

4 Centrifuge at 280 × g for 1 minute.

5 Place the midi plate on the preprogrammed thermal cycler and run the EPM AMP program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days.

Clean Up Amplified Enriched Library

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

Consumables

- RSB (Resuspension Buffer)
- AMPure XP beads
- Freshly prepared 80% ethanol (EtOH)
- 96-well Hard-Shell 0.3 ml PCR plate
- 96-well midi plate
- Microseal 'B' adhesive seals

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
RSB	2°C to 8°C	Bring to room temperature.
AMPure XP beads	2°C to 8°C	Bring to room temperature.

2 Prepare fresh 80% ethanol from absolute ethanol.

3 Label a new Hard-Shell PCR plate REL with a marker.

4 Label a new midi plate REC2 with a marker.

Procedure

1 Centrifuge at 280 × g for 1 minute.

2 Transfer 50 µl to the corresponding well of the REC2 plate.

3 Add 90 µl AMPure XP beads to each well.

4 Apply the seal and shake RAC2 at 1800 rpm for 1 minute.

5 Incubate at room temperature for 5 minutes.

6 Centrifuge at 280 × g for 1 minute.

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

8 Remove and discard all supernatant from each well.

9 Wash 2 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.

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

11 Air-dry on the magnetic stand for 5 minutes.

12 Remove from the magnetic stand.

13 Add 32 µl (8 µl for 1-plex) RSB to each well.

14 Apply the seal and shake at 1800 rpm for 1 minute.

15 Incubate at room temperature for 2 minutes.

16 Centrifuge at 280 × g for 1 minute.

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

18 Transfer 30 µl (7.5 µl for 1-plex) supernatant to the corresponding well of the REL plate.

SAFE STOPPING POINT

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

Check Enriched Libraries

Perform the following procedures to check the quality of the 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 quantification of DNA libraries.

1 Quantify the libraries according to the Illumina *Sequencing Library qPCR Quantification Guide (document # 11322363)* or fluorometric method.

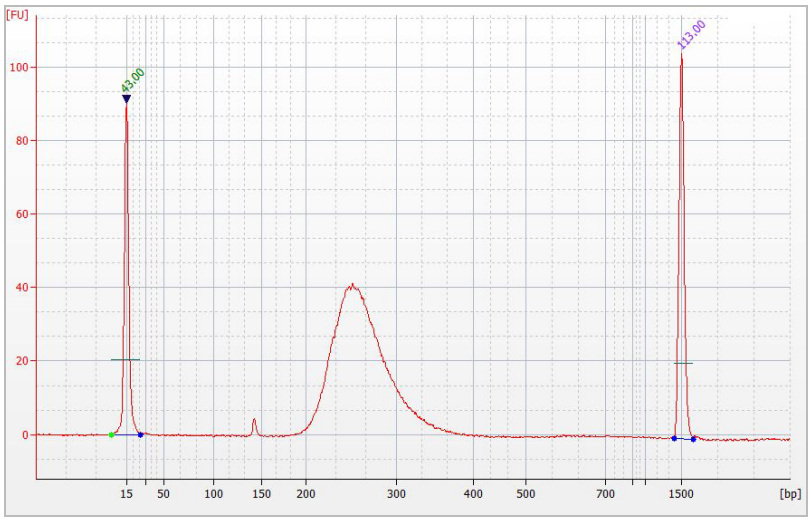
2 Quantify the libraries using qPCR (preferred method) according to the Illumina *Sequencing Library qPCR Quantification Guide (document # 11322363)* or fluorometric method.

Assess Quality **[Optional]**

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 ~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 Exome workflow Post-Enrichment Library Distribution



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

Supporting Information

Introduction

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

Product Contents

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

The following library prep, index adapter, enrichment, and coding exome oligo components are available to order through Illumina to support the TruSeq RNA Exome workflow.

Library Prep Component	Catalog #
TruSeq RNA Library Prep for Enrichment (48 samples)	20020189

Enrichment Component	Catalog #
TruSeq RNA Enrichment (12 multiplex enrichments, 48 single-plex enrichments)	20020490

Panel Component	Catalog #
Exome Panel (45 MB)	20020183

Index Adapter Component	Catalog #
TruSeq RNA Single Indexes (12 indexes, 24 samples) Set A	20020492
TruSeq RNA Single Indexes (12 indexes, 24 samples) Set B	20020493

TruSeq RNA Library Prep for Enrichment (48 Samples)

Library Prep for Enrichment, Store at -25°C to -15°C

Quantity	Reagent	Description
1	FSA	First Strand Synthesis Act D Mix
1	PMM	PCR Master Mix
1	PPC	PCR Primer Cocktail
1	SMM	Second Strand Marking Master Mix
1	EPH	Elution Primer Fragmentation Mix
1	LIG	Ligation Mix
1	ATL	A-Tailing Mix
1	STL	Stop Ligation Buffer
1	RSB	Resuspension Buffer

TruSeq RNA Enrichment (12 Enrichments)

This component is made up of two boxes with separate storage conditions. Sufficient reagents are provided to support 12 enrichments of 1-plex up to 4-plex.

Enrichment Reagents - Box 1, Store at 2°C to 8°C

Quantity	Reagent	Description
1	ET2	Elute Target Buffer 2
3	SMB	Streptavidin Magnetic Beads

Enrichment Component - Box 2, Store at -25°C to -15°C

Quantity	Reagent	Description
1	CT3	Capture Target Buffer 3
2	EE1	Enrichment Elution Buffer 1
4	EPM	Enhanced PCR Mix
2	EWS	Enrichment Wash Solution
1	HP3	2N NaOH
1	PPC	PCR Primer Cocktail
1	RSB	Resuspension Buffer

Coding Exome Oligos, Store at -25°C to -15°C

Quantity	Reagent	Description
4	CEX	Coding Exome Oligos

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.

Consumables and Equipment

Confirm that all required user-supplied consumables and equipment are present and available before starting the protocol.

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	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
96-well flat clear bottom black microplates (for 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
Conical centrifuge tubes (15 ml or 50 ml)	General lab supplier
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
96-well Hard-Shell 0.3 ml PCR 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 ultrapure water	General lab supplier

Consumable	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
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 - positive control] Human UHR total RNA	Agilent Technologies, part # 740000
[Optional - for starting material quality assessment] One of the following: • Standard Sensitivity RNA Analysis Kit (20nt Lower Marker) • Agilent RNA 6000 Nano Kit	 • Advanced Analytical Technologies, part # DNF-489 • Agilent Technologies, part # 5067-1511

Equipment

Equipment	Supplier/Description
DNA Engine Multi-Bay Thermal Cycler See *Thermal Cyclers* on page 36.	• Bio-Rad, part # PTC-0240G or • PTC-0220G, • with Alpha Unit, ALS-1296GC
Fluorometric quantitation with dsDNA binding dye reagents	General lab supplier
One of the following: • Fragment Analyzer Automated CE System • 2100 Bioanalyzer Desktop System	• Advanced Analytical Technologies, part # FSv2-CE2 or FSv2-CE10 • Agilent Technologies, part # G2940CA
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

Equipment	Supplier/Description
MIDI plate insert for heating system Note: Two inserts are recommended to support successive heating procedures.	Illumina, catalog # BD-60-601
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 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 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

Acronyms

Acronym	Definition
ALP	Adapter Ligation Plate
ATL	A-Tailing Mix
CAP	Clean Up ALP Plate
CCP	cDNA Clean Up Plate
CEX	Coding Exome Oligos
CPP	Clean Up PCR Plate
CT3	Capture Target Buffer 3
DFP	Depleted RNA Fragmentation Plate

Acronym	Definition
EE1	Enrichment Elution Buffer 1
EPH	Elute, Prime, Fragment High Mix
ET2	Elute Target Buffer 2
EWS	Enrichment Wash Solution
FSA	First Strand Synthesis Act D Mix
HP3	2N NaOH
IEM	Illumina Experiment Manager
LIG	Ligation Mix
LRM	Local Run Manager
PCR	Polymerase Chain Reaction Plate
PMM	PCR Master Mix
PPC	PCR Primer Cocktail
REA	RNA Exome Amplification Plate
REC1	RNA Exome Clean Up Plate 1
REC2	RNA Exome Clean Up Plate 2
REH1	RNA Exome Hyb Plate 1
REH2	RNA Exome Hyb Plate 2
REL	RNA Exome Library Plate
REW1	RNA Exome Wash Plate 1
REW2	RNA Exome Wash Plate 2
RSB	Resuspension Buffer
SMB	Streptavidin Magnetic Beads
SMM	Second Strand Marking Master Mix
STL	Stop Ligation Buffer
TSP	Target Sample Plate

Technical Assistance

For technical assistance, contact Illumina Technical Support.

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

Illumina Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
Australia	+1.800.775.688	
Austria	+43 800006249	+43 19286540
Belgium	+32 80077160	+32 34002973
China	400.066.5835	
Denmark	+45 80820183	+45 89871156
Finland	+358 800918363	+358 974790110
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Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

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