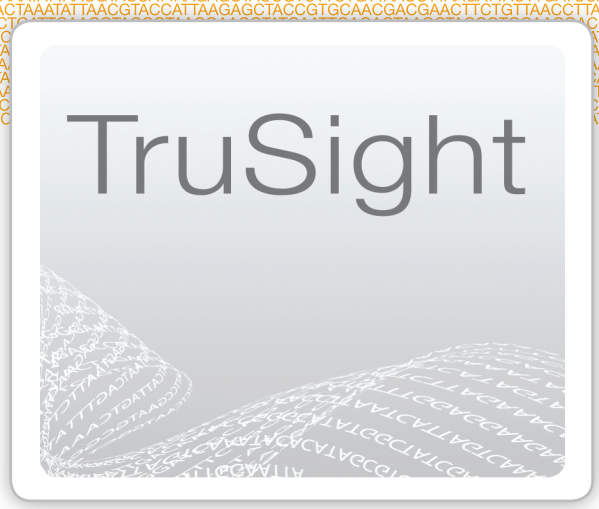
illumina®

TruSight® RNA Fusion Panel Reference Guide



For Research Use Only. Not for use in diagnostic procedures.

Document # 1000000009154 v00
July 2016

Customize a short end-to-end workflow guide with the Custom Protocol Selector
support.illumina.com/custom-protocol-selector.html

Revision History

Document	Date	Description of Change
Document # 1000000009154 v00	July 2016	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, and then selectively enrich for 507 genes that have been associated with gene fusions in cancer using reagents provided in an Illumina® TruSight® RNA Fusion Panel. The library is suitable for subsequent cluster generation and DNA sequencing.

The RNA is fragmented using divalent cations under elevated temperature. For degraded RNA samples, such as those from formalin-fixed paraffin-embedded (FFPE) tissues, fragmentation is omitted. The 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 expressed cancer-associated genes are captured from this library using sequence-specific probes to create the final library.

This library prep protocol offers:

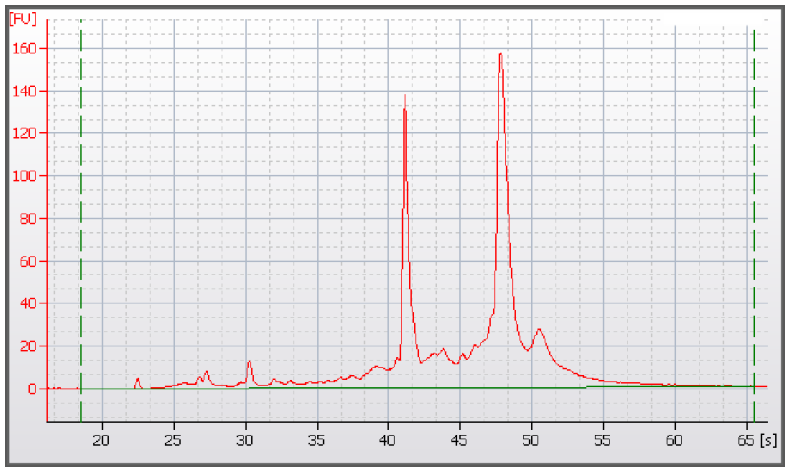
- High data quality even from degraded or FFPE-derived RNA samples
- Input requirement as low as 10 ng for fresh/frozen samples and 20 ng for FFPE samples
- Strand information on RNA transcripts

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 reduced sensitivity.
  - Use NanoDrop to determine FFPE RNA concentration.
- The protocol has been tested using 10 ng of high-quality universal human reference total RNA as input and 20–100 ng of total RNA extracted from FFPE.
  - Use of RNA from other tissues or lower quality RNA might require further optimization to determine the input amount.
- For FFPE or degraded samples, determine the quality of the RNA starting material.
  - 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.
  - 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 protocol has been tested using 20–100 ng of total RNA extracted from FFPE as input. The following input recommendations for FFPE RNA (20–100 ng) are provided as a guide for sample-specific optimization.

Table 1 FFPE RNA Input Recommendations

Quality	DV_{200}	Input Requirement Per Reaction
High	> 70%	20 ng
Medium	50–70%	20–50 ng
Low	30–50%	50–100 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.

- For samples that border a quality classification, err towards the higher end of the input recommendation.
- Poor results may be obtained with low quality FFPE samples.

NOTE
For more information, see the *Evaluating RNA Quality from FFPE Samples* tech note for the TruSight RNA Fusion Panel. See *Additional Resources* on page 6 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, plus a trace of FFPE quality that is not recommended for use with TruSight RNA Fusion Panel.

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

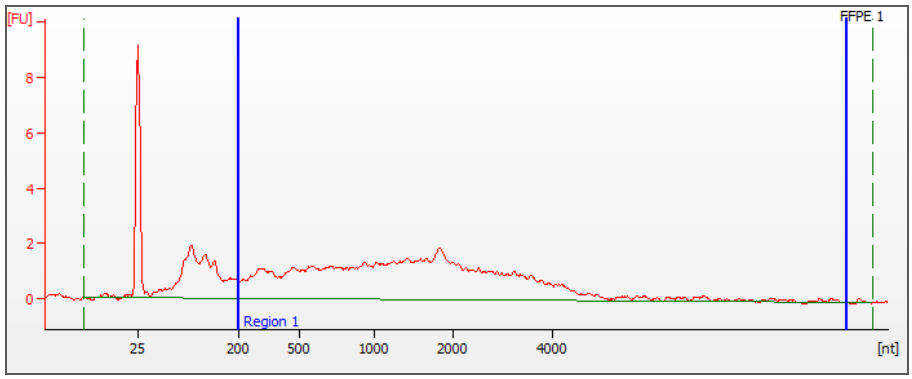


Figure 3 Example: Medium Quality FFPE (DV_{200} = 55%)

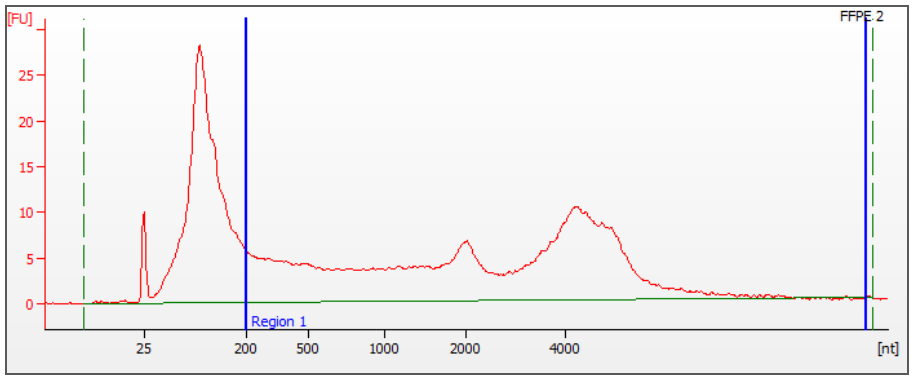


Figure 4 Example: Low Quality FFPE (DV_{200} = 30%)

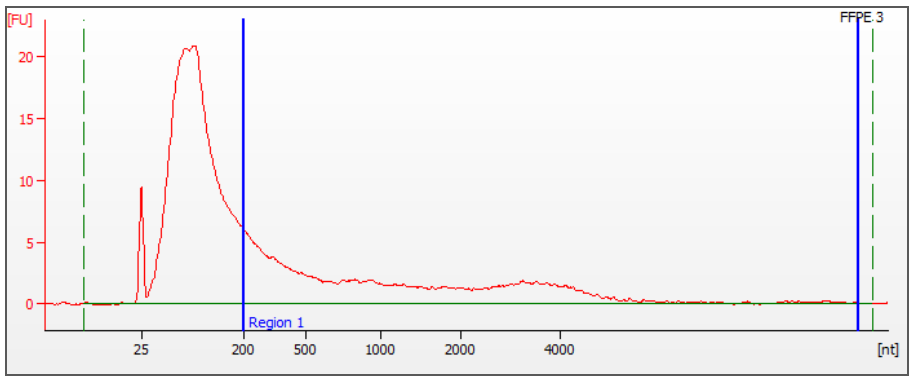
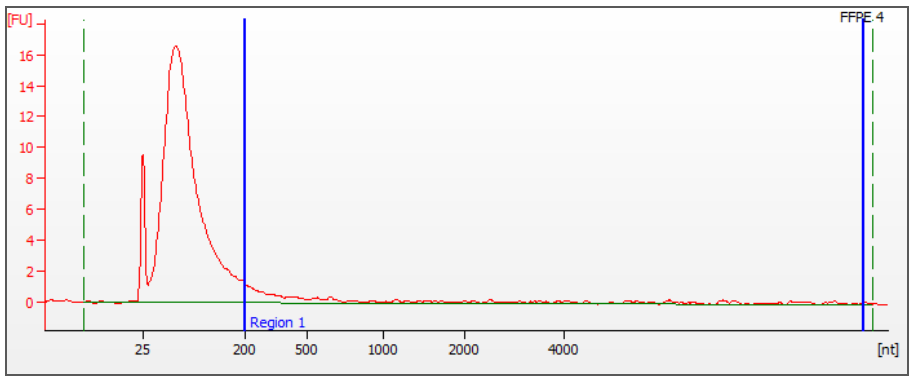


Figure 5 Example: FFPE Quality Not Recommended for Use (DV_{200} = 8%)



Positive Control

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

Additional Resources

Visit the TruSight RNA Fusion Panel support page on the Illumina website for documentation, software downloads, training resources, and information about compatible Illumina products.

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

Resource	Description
Custom Protocol Selector	support.illumina.com/custom-protocol-selector.html A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
TruSight RNA Fusion Panel Protocol Guide (document # 1000000009155)	Provides only protocol instructions. The protocol guide is intended for experienced users. For new or less experienced users, see the TruSight RNA Fusion Panel Reference Guide.
TruSight RNA Fusion Panel Checklist (document # 1000000009156)	Provides a checklist of the protocol steps. The checklist is intended for experienced users. For new or less experienced users, see the TruSight RNA Fusion Panel Reference Guide.
TruSight RNA Fusion Panel Consumables & Equipment (document # 1000000009157)	Provides an interactive checklist of user-provided consumables and equipment.
Evaluating RNA Quality from FFPE Samples tech note	Provides effectivity profiles for FFPE RNA.
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.
Local Run Manager RNA Fusion Analysis Module Workflow guide (document # 1000000010786)	Provides information about analyzing TruSight RNA Fusion libraries using the Local Run Manager RNA Fusion analysis module.
Illumina Experiment Manager Guide (document # 15031335) and *IEM TruSight RNA Pan-Cancer and TruSight RNA Fusion Quick Reference Card (document # 1000000014136)*	Provides information about creating and editing appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate.
BaseSpace help (help.basespace.illumina.com)	Provides information about the BaseSpace sequencing data analysis tool that also enables you to organize samples, libraries, pools, and sequencing runs in a single environment.

Protocol

Introduction

This chapter describes the TruSight RNA Fusion protocol.

- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- Review Best Practices from the TruSight RNA Fusion support page on the Illumina website.
- Before proceeding, confirm kit contents and make sure that you have the required equipment and consumables.

Prepare for Pooling

Use Local Run Manager, IEM, or BaseSpace to record information about your samples before beginning library preparation.

- Use Local Run Manager to record samples to be sequenced, specify sequencing run parameters, and run on-instrument analysis.
- Use IEM to create and edit sample sheets for Illumina sequencing systems and analysis software.
- Use the BaseSpace Prep tab to organize samples, libraries, pools, and a run for Illumina sequencing systems and analysis software.

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

Tips and Techniques

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

Avoiding Cross-Contamination

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

Sealing the Plate

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

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.
- If beads are aspirated into the pipette tips, dispense back to the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

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 RNA

- When harvesting total RNA, use a method that quickly disrupts tissue, isolates and stabilizes RNA, and includes a DNase removal step.
- Use sterile, RNase-free consumables. Designate a set of pipettes for RNA lab work and a set of microcentrifuge tubes for this protocol.
- Use a RNAse/DNAse decontamination solution to decontaminate work surfaces and equipment before starting this protocol.

Library Prep Workflow

The following diagram illustrates the workflow using a TruSight RNA Fusion Panel kit. Safe stopping points are marked between steps.

The times provided are based on batches of 8 samples.

Figure 6 TruSight RNA Fusion Panel Workflow

Fragment RNA

This process fragments and primes RNA for cDNA synthesis.

NOTE
If starting with FFPE RNA, see *Total RNA Input* on page 3 for more information. 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.

NOTE
If you are preparing FFPE and non-FFPE samples, do not transfer the FFPE samples to the PCR plate until after the non-FFPE samples are fragmented in step 5. EPH is added to all samples.

2 Add 8.5 µl EPH to each well.

3 Pipette to mix.

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

WARNING
If starting with FFPE RNA, do not perform the following incubation procedure. Proceed immediately to *Synthesize First Strand cDNA* on page 13.

5 Place on the preprogrammed thermal cycler and run the Elution 2-Frag-Prime program.

6 Centrifuge at 280 × g for 1 minute.

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)
- Protoscript II
- 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 49.

Preparation

1 Prepare the following consumables.

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

Procedure

1 Centrifuge FSA and Protoscript II at 600 × g for 5 seconds.

2 Add 50 µl Protoscript II to FSA. Pipette or invert to mix. Then apply the seal and centrifuge briefly.
Label the FSA tube to indicate that Protoscript II has been added.

NOTE
If you are not using the entire contents of FSA, add Protoscript II at a ratio of 1 µl Protoscript 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 Protoscript II and FSA mixture to each well.

4 Pipette to mix.

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

Synthesize Second Strand cDNA

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

Consumables

- RSB (Resuspension Buffer)
- SMM (Second Strand Marking Master Mix)
- AMPure XP Beads
- Freshly prepared 80% ethanol (EtOH)
- 96-well midi plate
- 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
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 Prepare fresh 80% ethanol from absolute ethanol.

3 Save the following Synthesize 2nd Strand program on the thermal cycler:
- Choose the preheat lid option and set to 30°C
- 16°C for 30 minutes
- Hold at 4°C
- Each well contains 50 µl

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 Pipette to mix.

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

6 Place on the preprogrammed thermal cycler and run the Synthesize 2nd Strand program.

7 Place on the bench and let stand to bring to room temperature (~5 minutes).

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 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 20 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 (~5 minutes).

17 Transfer 17.5 µl supernatant to the corresponding well of the ALP plate.

SAFE STOPPING POINT

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

Adenylate 3′ Ends

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

Consumables

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

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 Save the following ATAIL70 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 37°C for 30 minutes
- 70°C for 5 minutes
- Hold at 4°C
- Each well contains 30 µl

Procedure

1 Centrifuge ATL at 600 × g for 5 seconds.

2 Add 12.5 µl ATL to each well.

3 Pipette to mix.

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

5 Place on the thermal cycler and start the program ATAIL70.

6 Place on ice for 1 minute or until cooled to 2°C to 8°C.

Ligate Adapters

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

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 for 10 minutes. Return to storage after use.
STL	-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 Prepare fresh 80% ethanol from absolute ethanol.

3 Save the following LIG30 program on the thermal cycler:
- Choose the preheat lid option and set to 100°C
- 30°C for 10 minutes
- Hold at 4°C
- Each well contains 37.5 µl

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

5 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. Pipette to mix.
5. Apply the seal and centrifuge at 280 × g for 1 minute.
6. Place on the thermal cycler and start the program LIG30.
7. Centrifuge STL at 600 × g for 5 seconds.
8. Add 5 µl STL to each well.
9. Pipette to mix.
10. Apply the seal and centrifuge at 280 × g for 1 minute.

Clean Up Ligated Fragments

1. Add 42 µl AMPure XP Beads to each well of a new midi plate labeled CAP.
2. Transfer entire volume (42 µl) from ALP plate to CAP.
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 22.5 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 20 µl supernatant to the corresponding well of the PCR plate.

SAFE STOPPING POINT

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

Perform First PCR Amplification

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

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

Consumables

- PMM (PCR Master Mix)
- PPC (PCR Primer Cocktail)
- RSB (Resuspension Buffer)
- AMPure XP Beads
- Freshly prepared 80% ethanol (EtOH)
- 96-well midi plate
- 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
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 Prepare fresh 80% ethanol from absolute ethanol.

3 Save the following PMM AMP 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
4 Label a new midi plate PPP with a marker.
5 Label a new Hard-Shell PCR plate TSP1 with a marker.

Procedure

Amplify DNA Fragments

1 Place the PCR plate on ice and add 5 µl PPC to each well.
2 Add 25 µl PMM to each well.
3 Pipette to mix.

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

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

Clean Up Amplified DNA

1 Add 50 µl AMPure XP Beads to the PPP plate for each well corresponding to a sample in the PCR plate.
2 Apply the seal and centrifuge PCR plate at 280 × g for 1 minute.
3 Transfer the entire volume (50 µl) to the PPP plate.
4 Apply the seal and shake at 1800 rpm for 2 minutes.
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 12.5 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 (2–5 minutes).

18 Transfer 12 µl supernatant to the corresponding well of the TSP1 plate.

SAFE STOPPING POINT

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

Check Libraries

Quantify Library

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

1 If using a Standard Sensitivity NGS Fragment Analysis Kit on an Advanced Analytical Fragment Analyzer, run 2 µl undiluted 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 ~250–300 bp.

4 Calculate the concentration of the library using a region selection of 160–700 bp. A sharp peak observed at ~150 bp is indicative of unligated adapter. Do not include this peak in the determination of library concentration, as this peak is eliminated in subsequent capture steps.

Figure 7 Example Library Size Distribution

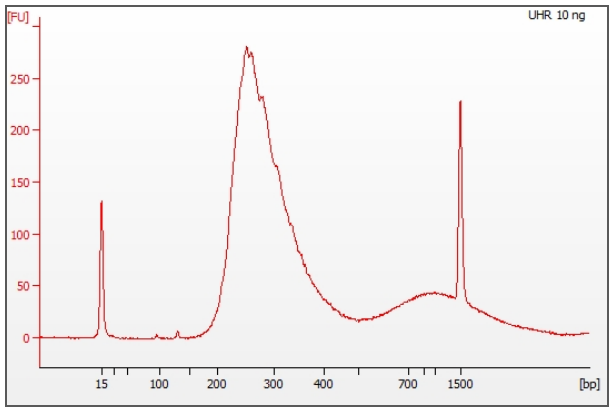
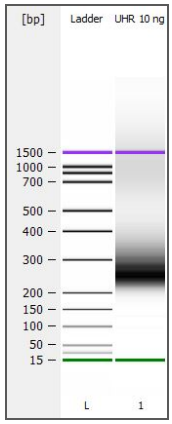


Figure 8 TruSight RNA Fusion PCR Product



Hybridize Probes

This step binds targeted regions of the library with capture probes.

Consumables

- CT3 (Capture Target Buffer 3)
- RFO (RNA Fusion Oligos)
- RSB (Resuspension Buffer)
- 96-well Hard-Shell 0.3 ml PCR plate
- Microseal 'B' adhesive seal

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
RFO	-25°C to -15°C	Thaw at room temperature.
CT3	-25°C to -15°C	Thaw at room temperature. Vortex before use.
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 90 minutes
- Hold at 58°C

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

3 Label a new Hard-Shell PCR plate RAH1 with a marker.

Procedure

1 Dilute 200 ng of each library in 10 µl RSB.

2 Add the following items in the order listed to each well of the RAH1 plate for a final volume of 25 µl.
- 200 ng of library (in 10 µl of RSB)
- CT3 (12.5 µl)
- RFO (2.5 µl)

3 Apply the seal and 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. Each well contains 25 µl. Remove from the thermal cycler immediately after the 90 minute incubation.

Capture 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 a second round of hybridization.

Consumables

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

About Reagents

- EEW is light sensitive. Prepare away from light.
- EEW can be cloudy after reaching room temperature.
- Make a fresh elution premix for each capture step. 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.
EEW	-25°C to -15°C	Thaw at room temperature. Vortex before use. 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. Invert and vortex to mix before use. Return to storage after use.

2 Save the following RNA BIND program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - 50°C for 20 minutes
   - Hold at 50°C

3 Label a new Hard-Shell PCR plate RAH2 with a marker.

Procedure

First Bind

1 Centrifuge RAH1 at 280 × g for 1 minute.

NOTE
Avoid significant sample loss. Poor plate sealing or insufficient locking or heating of the thermal cycler lid can cause evaporation of samples.

2 Add 62.5 µl SMB to each well of RAH1.

3 Apply the seal and shake at 1200 rpm for 5 minutes.

4 Incubate at room temperature for 25 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 (~85 µl).

8 Remove from the magnetic stand.

First Wash

1 Add 50 µl EEW to each well.

2 Apply the seal and centrifuge at 280 × g for 10 seconds to bring down beads from the sides of the wells.

3 Pipette entire volume up and down to mix, making sure that all beads are resuspended.

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

5 Place on the thermal cycler and start the program RNA BIND. Each well contains 52.5 µl.

6 After 20 minutes, immediately place on a magnetic stand and wait until the liquid is clear (~2 minutes).

7 Remove and discard all supernatant from each well (~50 µl).

8 Remove from the magnetic stand.

9 Repeat steps 1–8 for a total of 2 washes.

First Elution

1 Add the following items in a 1.7 ml microcentrifuge tube to create elution premix, and then vortex.
- EE1 (9.5 µl)
- HP3 (0.5 µl)

Create enough elution premix for the total number of samples to be processed, plus 10% excess volume.

2 Add 10 µl elution premix to each well.

3 Apply the seal and centrifuge at 280 × g for 10 seconds to bring down beads from the sides of the wells.

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 9 µl supernatant to the corresponding well of the RAH2 plate.

9 Add 1.7 µl ET2 to each well.

10 Apply the seal and 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 7 days.

Perform Second Hybridization

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

Consumables

- CT3 (Capture Target Buffer 3)
- RFO (RNA Fusion 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
RFO	-25°C to -15°C	Thaw at room temperature.
CT3	-25°C to -15°C	Thaw at room temperature. Vortex before use.
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 in the RAH2 plate that contains a sample for a total volume of 25.7 µl.
   - CT3 (12.5 µl)
   - RFO (2.5 µl)

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

3 Centrifuge at 280 × g for 1 minute.

4 Place on the preprogrammed thermal cycler and run the RNA HYB program. Each well contains 25.7 µl.
   Remove from the thermal cycler immediately after the 90 minute incubation.

Perform Second Capture

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)
- EEW (Enhanced Enrichment Wash Buffer)
- HP3 (2 N NaOH)
- SMB (Streptavidin Magnetic Beads)
- 96-well midi plate
- 1.7 ml microcentrifuge tube
- Microseal 'B' adhesive seals

About Reagents

- EEW is light sensitive. Prepare away from light.
- EEW can be cloudy after reaching room temperature.
- Make a fresh elution premix for each capture step. 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.
EEW	-25°C to -15°C	Thaw at room temperature. Vortex EEW before use. 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. Invert and vortex SMB to mix before use. Return to storage after use.

2 Label a new midi plate RAW1 with a marker.

Procedure

Second Bind

1 Centrifuge RAH2 at 280 × g for 1 minute.

NOTE
Avoid significant sample loss. Poor plate sealing or insufficient locking or heating of the thermal cycler lid can cause evaporation of samples.

2 Add 62.5 µl SMB to each well.

3 Apply the seal and shake at 1200 rpm for 5 minutes.

4 Incubate at room temperature for 25 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 (~85 µl).

8 Remove from the magnetic stand.

Second Wash

1 Add 50 µl EEW to each well.

2 Apply the seal and centrifuge at 280 × g for 10 seconds to bring down beads from the sides of the wells.

3 Pipette entire volume up and down to mix, making sure that all beads are resuspended.

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

5 Place on the thermal cycler and start the program RNA BIND. Each well contains 53.2 µl.

6 After 20 minutes, immediately place on a magnetic stand and wait until the liquid is clear (~2 minutes).

7 Remove and discard all supernatant from each well (~50 µl).

8 Remove from the magnetic stand.

9 Repeat steps 1–8 for a total of 2 washes.

Second Elution

1 Add the following items in a 1.7 ml microcentrifuge tube to create elution premix, and then vortex.
   - EE1 (9.5 µl)
   - HP3 (0.5 µl)

   Create enough elution premix for the total number of samples to be processed, plus 10% excess volume.

2 Add 10 µl elution premix to each well.

3 Apply the seal and centrifuge at 280 × g for 10 seconds to bring down beads from the sides of the wells.

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 9 µl supernatant to the corresponding well of the RAW1 plate.

9 Add 1.7 µl ET2 to each well.

10 Apply the seal and shake at 1200 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	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 Prepare fresh 80% ethanol from absolute ethanol.

3 Label a new Hard-Shell PCR plate PCR2 with a marker.

Procedure

1 Add 20 µl AMPure XP Beads to each well of the RAW1 plate.

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

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 27.5 µl 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 2 minutes.

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 PCR2 plate.

SAFE STOPPING POINT

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

Perform Second PCR Amplification

This step uses a 14-cycle PCR program to amplify the enriched library.

Consumables

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

About Reagents

- Avoid repeated freeze-thaw cycles of EPM and PPC.

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
- 14 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 Add 5 µl PPC to each well.

2 Add 20 µl EPM to each well.

3 Pipette to mix.

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

5 Place 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	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 Prepare fresh 80% ethanol from absolute ethanol.

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

4 Label a new midi plate RAC2 with a marker.

Procedure

1 Centrifuge the PCR2 plate at 280 × g for 1 minute.

2 Add 90 µl AMPure XP Beads to each well of the RAC2 plate.

3 Transfer 50 µl from the PCR2 plate to the corresponding well of the RAC2 plate.

4 Apply the seal and shake RAC2 at 1800 rpm for 2 minutes.

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 140 µl 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 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 supernatant to the corresponding well of the RAL plate.

SAFE STOPPING POINT

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

Check Enriched Libraries

Perform the following procedures to check enriched library quality.

Quantify Libraries

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

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

Check Library Quality

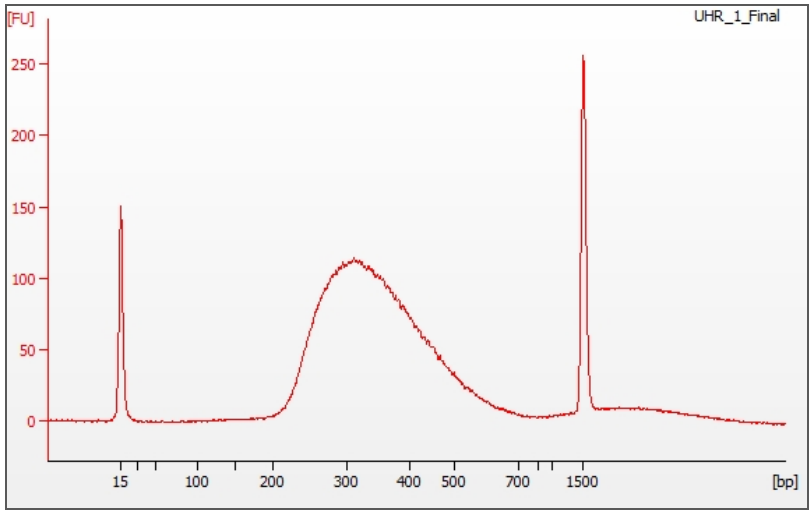
1 If using a Standard Sensitivity NGS Fragment Analysis Kit on an Advanced Analytical Fragment Analyzer, run 2 µl of the postenriched library.

2 If using a DNA 1000 Chip, run 1 µl of the postenriched library.

3 Check the size and purity of the sample. Expect the final product to be a band at ~250–300 bp.

4 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.

Figure 9 Example TruSight RNA Fusion Postenrichment Library Distribution



5 Denature and dilute pooled libraries to the loading concentration for the instrument you are using. For loading recommendations, see the TruSight RNA Fusion Panel support page.

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 the required consumables and equipment.

Acronyms

Acronym	Definition
ALP	Adapter Ligation Plate
ATL	A-Tailing Mix
CAP	Clean Up ALP Plate
CCP	cDNA Clean Up Plate
CPP	Clean Up PCR Plate
CT3	Capture Target Buffer 3
DFP	Depleted RNA Fragmentation Plate
EE1	Enrichment Elution Buffer 1
EEW	Enhanced Enrichment Wash Buffer
EPH	Elute, Prime, Fragment High Mix
EPM	Enhanced PCR Mix
ET2	Elute Target Buffer 2
FSA	First Strand Synthesis Act D Mix
HP3	2N NaOH
LIG	Ligation Mix
PCR	Polymerase Chain Reaction Plate
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
RFO	RNA Fusion Oligos
RSB	Resuspension Buffer
SMB	Streptavidin Magnetic Beads
SMM	Second Strand Marking Master Mix

Acronym	Definition
STL	Stop Ligation Buffer
TSP	Target Sample Plate

Kit Contents

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

The TruSight RNA Fusion Panel (48 samples) is available in a Set A and a Set B. Each 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.

Kit Name	Catalog #	Number of Samples Supported	Number of Indexes
TruSight RNA Fusion Panel - Set A	RS-304-1002	48	12
TruSight RNA Fusion Panel - Set B	RS-304-1003	48	12

Box 1, Store at -25°C to -15°C

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

Box 2, Set A or Set B, Store at -25°C to -15°C

You receive either set A or B, depending on the kit.

Table 2 Set A

Quantity	Reagent	Description
1	AR002	RNA Adapter Index 2
1	AR004	RNA Adapter Index 4
1	AR005	RNA Adapter Index 5
1	AR006	RNA Adapter Index 6
1	AR007	RNA Adapter Index 7
1	AR012	RNA Adapter Index 12
1	AR013	RNA Adapter Index 13
1	AR014	RNA Adapter Index 14
1	AR015	RNA Adapter Index 15
1	AR016	RNA Adapter Index 16
1	AR018	RNA Adapter Index 18
1	AR019	RNA Adapter Index 19
1	RSB	Resuspension Buffer
1	EPH	Elute, Prime, Fragment High Mix
1	LIG	Ligation Mix
1	ATL	A-Tailing Mix
1	STL	Stop Ligation Buffer

Table 3 Set B

Quantity	Reagent	Description
1	AR001	RNA Adapter Index 1
1	AR003	RNA Adapter Index 3
1	AR008	RNA Adapter Index 8
1	AR009	RNA Adapter Index 9
1	AR010	RNA Adapter Index 10
1	AR011	RNA Adapter Index 11
1	AR020	RNA Adapter Index 20
1	AR021	RNA Adapter Index 21
1	AR022	RNA Adapter Index 22
1	AR023	RNA Adapter Index 23
1	AR025	RNA Adapter Index 25
1	AR027	RNA Adapter Index 27
1	RSB	Resuspension Buffer
1	EPH	Elute, Prime, Fragment High Mix
1	LIG	Ligation Mix
1	ATL	A-Tailing Mix
1	STL	Stop Ligation Buffer

Box 3, Store at 2°C to 8°C

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

Box 4, Store at -25°C to -15°C

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

Box 5, Oligos, Store at -25°C to -15°C

Quantity	Reagent	Description
1	RFO	RNA Fusion Oligos

Consumables and Equipment

Make sure that you have the required user-supplied consumables and equipment 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
1.7 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 single channel pipettes	General lab supplier
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
KAPA Library Quantification Complete Kit (Universal)	Kapa Biosystems, part # KK4824
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 8-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
Protoscript II Reverse Transcriptase	NEB, part # M0368L
[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 47.	• Bio-Rad, part # PTC-0240G or • PTC-0220G, with Alpha Unit, ALS-1296GC
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
Vortexer	General lab supplier

Thermal Cyclers

The following table lists the recommended settings for the thermal cycler and 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

Index Adapter Tube Sequences

The kit contains the following index adapter sequences.

- The index numbering is not contiguous. There is no Index 17, 24, or 26.
- The sequence contains 7 bases. The seventh base, shown in parenthesis (), is not included in the Index Read. Record only the first 6 bases in a sample sheet. For indexes 13 and above, the seventh base (in parentheses) might not be A, which is seen in cycle 7 of the Index Read.
- For more information on the number of cycles used to sequence the Index Read, see the system guide for your Illumina sequencing platform.

Table 4 LT Set A Index 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 5 LT Set B Index 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 6 Illumina General Contact Information

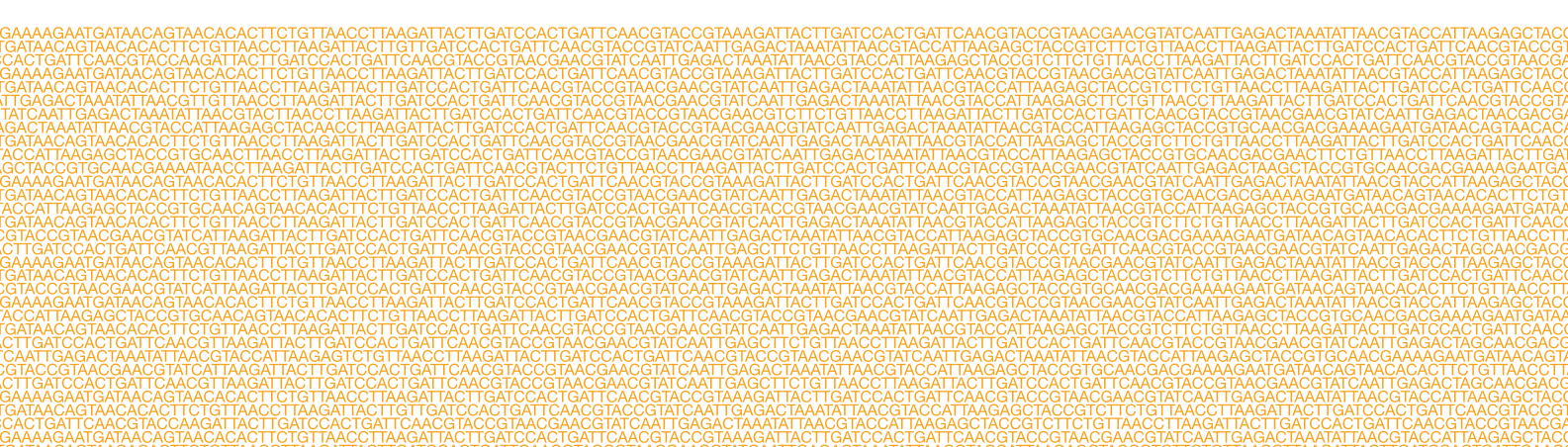
Website	www.illumina.com
Email	techsupport@illumina.com

Table 7 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Japan	0800.111.5011
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
Belgium	0800.81102	Norway	800.16836
China	400.635.9898	Singapore	1.800.579.2745
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	Taiwan	00806651752
Hong Kong	800960230	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000
Italy	800.874909		

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

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



Illumina
5200 Illumina Way
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com