SureSelect XT HS2 mRNA Library Preparation System

Poly-A Selection and Strand-Specific mRNA Seq Library Preparation for the Illumina Platform

Protocol

Version A1, September 2020

SureSelect platform manufactured with Agilent SurePrint Technology

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Acknowledgment

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In this Guide...

This guide provides an optimized protocol for preparation of Illumina paired-end multiplexed mRNA sequencing libraries.

1 Before You Begin

This chapter contains information that you should read and understand before you start an experiment.

2 Fragmentation of Input RNA and Conversion to cDNA

This chapter describes the steps to enrich total RNA samples for poly-A mRNA, fragment the RNA samples, and then convert the RNA to cDNA fragments.

3 Library Preparation

This chapter describes the steps to prepare dual-indexed, duplex molecular-barcoded cDNA sequencing libraries.

4 Guidelines for Multiplexed Sequencing

This chapter provides guidelines for sequencing sample preparation and processing using Illumina NGS platforms.

5 Reference

This chapter contains reference information, including component kit contents and index sequences.

What's New in Version A1

- Updates to index pair sequence tables (page 59 through page 66) including updates to P5 index platform descriptions and correction of well position typographical errors
- Updates to downstream sequencing support information (see Table 27 on page 47 and *Note* on page 58)

Content

1 Before You Begin

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

NOTE Agilent guarantees performance and provides technical support for the SureSelect reagents required for this workflow only when used as directed in this Protocol.

Overview of the Workflow

The SureSelect XT HS2 mRNA workflow for the preparation of NGS-ready libraries is summarized in Figure 1.

Figure 1 Overall mRNA sequencing sample preparation workflow.

Procedural Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Use best-practices to prevent PCR product and ribonuclease contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow.
  2. Maintain clean work areas. Clean the surfaces that pose the highest risk of contamination daily using a 10% bleach solution, or equivalent.
  3. Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
  4. Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.
- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of domed caps. Cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler and from other procedural steps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during thermal cycler incubation steps.
- In general, follow Biosafety Level 1 (BSL1) safety rules.
- Possible stopping points, where samples may be stored at 4°C or –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.

Safety Notes

CAUTION

- Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Materials Required

Materials required to complete the SureSelect XT HS2 mRNA protocol are listed in the tables in this section. Select the preferred SureSelect XT HS2 mRNA Reagent Kit format from Table 1, and refer to Table 2 through Table 4 for additional materials needed to complete the protocols.

Table 1 SureSelect XT HS2 mRNA Library Preparation Kit Varieties

Description	Kit Part Number	
	16 Reaction Kit*	96 Reaction Kit†
SureSelect XT HS2 mRNA Library Preparation Kit	G9995A (with Index Pairs 1–16)	G9997A (with Index Pairs 1–96) G9997B (with Index Pairs 97–192) G9997C (with Index Pairs 193–288) G9997D (with Index Pairs 289–384)
SureSelect XT HS2 mRNA Library Preparation Kit with AMPure® XP Beads‡	G9996A (with Index Pairs 1–16)	G9998A (with Index Pairs 1–96) G9998B (with Index Pairs 97–192) G9998C (with Index Pairs 193–288) G9998D (with Index Pairs 289–384)

* 16-reaction kits contain enough reagents for 2 runs containing 8 samples per run.

† 96-reaction kits contain enough reagents for 4 runs containing 24 samples per run.

‡ AMPure, Beckman, and Beckman Coulter are trademarks or registered trademarks of Beckman Coulter, Inc.

Table 2 Required Reagents

Description	Vendor and Part Number	Notes
1X Low TE Buffer	Thermo Fisher Scientific p/n 12090-015, or equivalent	10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276	—
Nuclease-free Water	Thermo Fisher Scientific p/n AM9930	Water should not be DEPC-treated
AMPure® XP Kit 5 ml 60 ml 450 ml	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882	Separate purchase **not** required for use with SureSelect XT HS2 mRNA Reagent Kits that include SureSelect RNA AMPure® XP Beads (Agilent p/n G9996A, G9998A, G9998B, G9998C, or G9998D)
QPCR Human Reference Total RNA	Agilent p/n 750500	Control input RNA (optional)

Table 3 Required Equipment

Description	Vendor and Part Number
Thermal Cycler with 96-well, 0.2 ml block	Various suppliers
Plasticware compatible with the selected thermal cycler: 96-well plates or 8-well strip tubes Tube cap strips	Consult the thermal cycler manufacturer's recommendations
Low-adhesion tubes (RNase, DNase, and DNA-free) 1.5 mL 0.5 mL	USA Scientific p/n 1415-2600 p/n 1405-2600
Microcentrifuge	Eppendorf microcentrifuge, model 5417C or equivalent
Plate or strip tube centrifuge	Labnet International MPS1000 Mini Plate Spinner, p/n C1000 (requires adapter, p/n C1000-ADAPT, for use with strip tubes) or equivalent
Small-volume spectrophotometer	NanoDrop 2000, Thermo Fisher Scientific p/n ND-2000 or equivalent
Multichannel pipette	Rainin Pipet-Lite Multi Pipette or equivalent
Single channel pipettes (10-, 20-, 200-, and 1000-µl capacity)	Rainin Pipet-Lite Pipettes or equivalent
Sterile, nuclease-free aerosol barrier pipette tips	general laboratory supplier
Vortex mixer	general laboratory supplier
Ice bucket	general laboratory supplier
Powder-free gloves	general laboratory supplier
Magnetic separator	Thermo Fisher Scientific p/n 12331D or equivalent*

* Select a magnetic separator configured to collect magnetic particles on one side of each well. Do not use a magnetic separator configured to collect the particles in a ring formation.

Table 4 Nucleic Acid Analysis Platform Options--Select One

Description	Vendor and Part Number
Agilent 4200 TapeStation	Agilent p/n G2991AA
Consumables:	
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
RNA ScreenTape	Agilent p/n 5067-5576
RNA ScreenTape Sample Buffer	Agilent p/n 5067-5577
RNA ScreenTape Ladder	Agilent p/n 5067-5578
High Sensitivity RNA ScreenTape	Agilent p/n 5067-5579
High Sensitivity RNA ScreenTape Sample Buffer	Agilent p/n 5067-5580
High Sensitivity RNA ScreenTape Ladder	Agilent p/n 5067-5581
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
Consumables:	
RNA 6000 Pico Kit	Agilent p/n 5067-1513
RNA 6000 Nano Kit	Agilent p/n 5067-1511
DNA 1000 Kit	Agilent p/n 5067-1504
Agilent 5200/5300/5400 Fragment Analyzer Instrument	Agilent p/n M5310AA/M5311AA/M5312AA
Consumables:	
RNA Kit (15NT)	p/n DNF-471-0500
HS RNA Kit (15NT)	p/n DNF-472-0500
NGS Fragment Kit (1-6000 bp)	p/n DNF-473-0500

Optional Materials

Table 5 Supplier Information for Optional Materials

Description	Vendor and Part Number	Purpose
Tween 20	Sigma-Aldrich p/n P9416-50ML	Sequencing library storage (see page 45)
MicroAmp Clear Adhesive Film	Thermo Fisher Scientific p/n 4311971	Improved sealing for flat strip caps
PlateLoc Thermal Microplate Sealer with Small Hotplate and Peelable Aluminum Seal for PlateLoc Sealer	Please contact the SureSelect support team (see page 2) or your local representative for ordering information	Sealing wells for protocol steps performed inside or outside of the thermal cycler

2 Fragmentation of Input RNA and Conversion to cDNA

This chapter describes the steps to prepare input RNA samples, including mRNA enrichment and RNA fragmentation, and the steps to convert the RNA fragments to strand-specific cDNA prior to sequencing library preparation.

The protocol is compatible with intact RNA prepared from fresh or fresh frozen samples. This protocol is not recommended for FFPE-derived RNA samples.

Protocol steps in this section use the components listed in Table 6 and Table 7 on page 16. Before you begin the protocol, equilibrate the components in Table 6 to room temperature and thaw the components in Table 7 on ice. Mix each component as directed before use (see the *Where Used* column).

Remove the AMPure XP beads from cold storage and equilibrate to room temperature for at least 30 minutes in preparation for use on page 25. *Do not freeze the beads at any time.*

Table 6 Reagents brought to room temperature before use in mRNA enrichment protocol

Kit Component	Storage Location	Where Used
Oligo(dT) Microparticles (tube with brown cap or bottle)	SureSelect Poly-A Selection Module (Pre PCR), 4°C	page 19
Bead Washing Buffer (bottle)	SureSelect Poly-A Selection Module (Pre PCR), 4°C	page 20
Bead Elution Buffer (tube with green cap or bottle)	SureSelect Poly-A Selection Module (Pre PCR), 4°C	page 20
Bead Binding Buffer (tube with purple cap or bottle)	SureSelect Poly-A Selection Module(Pre PCR), 4°C	page 20

Table 7 Reagents thawed and held on ice before use in fragmentation and cDNA synthesis steps

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
2X Priming Buffer (tube with purple cap)	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	page 22
First Strand Master Mix (amber tube with amber cap)*	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice for 30 minutes then keep on ice	Vortexing	page 23
Second Strand Enzyme Mix (tube with blue cap or bottle)	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	page 24
Second Strand Oligo Mix (tube with yellow cap)	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	page 24

* The First Strand Master Mix contains actinomycin-D and is provided ready-to-use. Keep the reagent in the supplied amber vial to protect the contents from exposure to light.

Step 1. Prepare and assess quality of total RNA

Before you begin, prepare total RNA from each sample in the run in nuclease-free water. The library preparation protocol requires 10 ng to 1 µg high-quality total RNA. A minimum RNA input of 50 ng is required for samples with reduced RNA integrity, as detailed below.

NOTE The protocol in this publication is suitable for intact RNA prepared from fresh or fresh frozen samples. This protocol is not recommended for FFPE-derived RNA samples.

Consider preparing an additional sequencing library in parallel, using a high-quality control RNA sample, such as Agilent's QPCR Human Reference Total RNA (p/n 750500). Use of this control is especially recommended during the first run of the protocol, to verify that all protocol steps are being successfully performed. Routine use of this control is helpful for any required troubleshooting, in order to differentiate any performance issues related to RNA input from other factors.

1. Prepare total RNA for each sample in the run in nuclease-free water.
2. Determine the RNA concentration using a small volume spectrophotometer. Verify that the 260/280 and 260/230 absorbance ratio values for the sample are both approximately 1.8 to 2.0. A significant deviation from ratios of 2.0 indicates that the sample may require further purification before use in NGS library preparation.
3. Analyze RNA integrity by determining the RNA Integrity Number (RIN), or equivalent, using one of the RNA qualification platforms listed in Table 4 on page 12. The RIN/RIN^e/RQN quality scores reported by these Agilent platforms are equivalent measures of RNA quality. Select the specific RNA assay for your platform based on the concentration determined in step 2.

   For optimal performance, total RNA samples should have RIN>8. For samples with RIN>8, the amount of total RNA needed for the library preparation protocol is 10 ng to 1 µg.

   Samples with RIN of 6 to 8 may be used in the protocol, using a minimum RNA input of 50 ng.

NOTE Libraries prepared using lower-quality RNA samples with RIN of 6 to 8 also require an additional purification step at a later stage of the protocol (see page 33).

Samples with RIN<6 are not suitable for use in this protocol; instead consider Agilent's SureSelect XT HS2 RNA system (see publication G9989-90000 at www.agilent.com).

4 Place each RNA sample in a separate well of a 96-well plate or strip tube in 25 µl of nuclease-free water.

Step 2. Select poly-A mRNA from total RNA

In this step, the samples are selectively enriched for poly-A tailed mRNA using two serial rounds of binding to oligo(dT) magnetic particles.

1. Vortex the Oligo(dT) Microparticles until the suspension appears homogeneous and consistent in color. If bead aggregates are still present after vortexing, mix thoroughly by pipetting up and down until the suspension appears homogeneous.
2. Add 25 µl of the homogeneous Oligo(dT) bead suspension to each total RNA sample well.
3. Seal the wells, then gently vortex for 5 seconds and briefly spin the plate or strip tube in a centrifuge or mini-plate spinner to collect the liquid without pelleting the beads.
4. Incubate the plate or strip tube in the thermal cycler and run the program in Table 8 to denature the RNA.

Table 8 Thermal cycler program for RNA denaturation*

Step	Temperature	Time
Step 1	65°C	5 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

* Use a reaction volume setting of 50 µl, if required for thermal cycler set up.

NOTE When using the SureCycler 8800 thermal cycler, the heated lid may be left on (default setting) throughout the library preparation incubation steps. The heated lid must be on during the amplification step on page 37.

5. After the thermal cycler reaches the 4°C Hold step, remove the plate or strip tube and incubate at room temperature for 5 minutes, to allow poly-A mRNA binding to the oligo(dT) beads.
6. Move the plate or strip tube to a magnetic separation device at room temperature. Wait for the solution to clear (approximately 2 to 5 minutes).

7 While keeping the plate or strip tube in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.

8 Remove the plate or strip tube from the magnetic stand. Gently add 200 µl of Bead Washing Buffer to each well.

Mix by pipetting up and down 10 times, using a P200 pipette set to 150 µl, without introducing bubbles.

CAUTION The Bead Washing Buffer contains detergent. It is important to process mixtures of the beads with the wash buffer without introducing bubbles or foam. If bubbles or foam are present during the wash steps, briefly spin the plate or strip tube in a centrifuge before continuing.

9 Place the plate or strip tube in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).

10 While keeping the plate or strip tube in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.

11 Remove the plate or strip tube from the magnetic stand. Add 25 µl of Bead Elution Buffer to each sample well.

12 Seal the wells, then gently vortex for 5 seconds. Briefly spin the plate or strip tube in a centrifuge or mini-plate spinner to collect the liquid.

13 Incubate the plate or strip tube in the thermal cycler and run the program in Table 9.

Table 9 Thermal cycler program for RNA elution*

Step	Temperature	Time
Step 1	80°C	2 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

* Use a reaction volume setting of 25 µl, if required for thermal cycler set up.

14 After the thermal cycler reaches the 4°C Hold step, remove the plate or strip tube and add 25 µl of Bead Binding Buffer to each sample well.

15 Seal the wells, then gently vortex for 5 seconds. Briefly spin the plate or strip tube in a centrifuge or mini-plate spinner to collect the liquid.

16 Incubate the samples at room temperature for 5 minutes, to allow poly-A mRNA to re-bind the beads.

17 Place the plate or strip tube in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).

18 While keeping the plate or strip tube in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.

19 Remove the plate or strip tube from the magnetic stand. Gently add 200 µl of Bead Washing Buffer to each well.

Mix by pipetting up and down 10 times, using a P200 pipette set to 150 µl, without introducing bubbles. If bubbles or foam are present, spin the plate or strip tube briefly before continuing.

20 Place the plate or strip tube in the magnetic stand at room temperature. Wait for the solution to clear (at least 2 minutes).

21 While keeping the plate or strip tube in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch or disturb the beads while removing the solution.

22 Remove the bead-bound RNA sample plate or strip tube from the magnetic stand. Add 10 µl of nuclease-free water to each sample well and keep the samples on ice.

23 Proceed immediately to "Step 3. Fragment the mRNA samples" on page 22.

Step 3. Fragment the mRNA samples

In this step, the poly-A mRNA-enriched samples are chemically-fragmented by treatment with metal ions at elevated temperature to a size appropriate for RNA sequencing library preparation. The 2X Priming Buffer used to resuspend the RNA-bound beads in this step includes both fragmentation agents and primers used for cDNA synthesis in the following steps. The fragmentation conditions shown in this section are appropriate for both 2 x 100 bp and 2 x 150 bp NGS read-length workflows.

1 Preprogram a thermal cycler with the program in Table 10. Immediately pause the program, and keep paused until samples are loaded in step 4.

Table 10 Thermal cycler program for fragmentation/elution of mRNA samples*

Step	Temperature	Time
Step 1	94°C	4 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

* Use a reaction volume setting of 20 µl, if required for thermal cycler set up.

2 Add 10 µl of 2X Priming Buffer to each sample well containing 10 µl of bead-bound RNA.

3 Cap the wells and vortex at high speed to resuspend the beads. Spin briefly to collect the liquid and remove bubbles.

4 Place the samples in the thermal cycler, and resume the thermal cycling program in Table 10. During this step the RNA is simultaneously fragmented and eluted from the oligo(dT) beads.

5 Once the thermal cycler program in Table 10 reaches the 4°C Hold step, transfer the fragmented RNA sample plate or strip tube from the thermal cycler to the magnetic stand at room temperature. Watch for the bead suspension to clear, then transfer each supernatant (approximately 20 µl) to wells of a fresh plate or strip tube. Keep the eluted RNA samples on ice. You can discard the oligo(dT) beads at this time.

   Minimize the processing time at this step to avoid rebinding of RNA to the beads.

Step 4. Synthesize first-strand cDNA

CAUTION

The First Strand Master Mix used in this step is viscous. Mix thoroughly by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining with other solutions. Pipetting up and down is not sufficient to mix this reagent.

The First Strand Master Mix is provided with actinomycin-D already supplied in the mixture. Do not supplement with additional actinomycin-D.

1 Preprogram a thermal cycler with the program in Table 11. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 11 Thermal cycler program for first-strand cDNA synthesis*

Step	Temperature	Time
Step 1	25°C	10 minutes
Step 2	37°C	40 minutes
Step 3	4°C	Hold

* Use a reaction volume setting of 28 µl, if required for thermal cycler set up.

2 Vortex the thawed vial of First Strand Master Mix for 5 seconds at high speed to ensure homogeneity.

3 Add 8.5 µl of First Strand Master Mix to each RNA sample well.

4 Mix well by pipetting up and down 15–20 times or seal the wells and vortex at high speed for 5–10 seconds. Spin briefly to collect the liquid.

5 Place the samples in the thermal cycler and resume the program in Table 11.

Step 5. Synthesize second-strand cDNA

CAUTION The Second Strand Enzyme Mix used in this step is viscous. Mix thoroughly by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining with other solutions. Pipetting up and down is not sufficient to mix this reagent.

1 Once the thermal cycler program in Table 11 begins the 4°C hold step, transfer the samples to ice.

2 Preprogram the thermal cycler with the program in Table 12. Immediately pause the program, and keep paused until samples are loaded in step 7.

Table 12 Thermal cycler program for second-strand synthesis*

Step	Temperature	Time
Step 1	16°C	60 minutes
Step 2	4°C	Hold

* Use a reaction volume setting of 58 µl, if required for thermal cycler set up.

3 Vortex the thawed vials of Second Strand Enzyme Mix and of Second Strand Oligo Mix at high speed for 5 seconds to ensure homogeneity.

4 Add 25 µl of Second Strand Enzyme Mix to each sample well. Keep on ice.

5 Add 5 µl of Second Strand Oligo Mix to each sample well, for a total reaction volume of 58.5 µl. Keep on ice.

6 Mix well by pipetting up and down 15–20 times or seal the wells and vortex at high speed for 5–10 seconds. Spin briefly to collect the liquid.

7 Place the plate or strip tubes in the thermal cycler and resume the program in Table 12.

Step 6. Purify cDNA using AMPure XP beads

1 Verify that the AMPure XP beads have been held at room temperature for at least 30 minutes before use.

2 Prepare 400 µl of 70% ethanol per sample, plus excess, for use in step 9.

NOTE The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day.

3 Mix the bead suspension well so that the suspension appears homogeneous and consistent in color.

4 Transfer the samples in the PCR plate or strip tube to room temperature, then add 105 µl of the homogeneous bead suspension to each cDNA sample well.

5 Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix. If the beads have splashed into the well caps, spin briefly to collect the samples, being careful not to pellet the beads.

6 Incubate samples for 5 minutes at room temperature.

7 Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 2 to 5 minutes).

8 Keep the plate or strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.

9 Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 µl of fresh 70% ethanol in each sample well.

10 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.

11 Repeat step 9 and step 10 once for a total of two washes.

12 Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol. Return the plate or strip tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

13 Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (up to 2 minutes).

NOTE Do not dry the bead pellet to the point that the pellet appears cracked during any of the bead drying steps in the protocol. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

14 Add 52 µl nuclease-free water to each sample well.

15 Seal the wells with strip caps, then vortex the plate or strip tube for 5 seconds. Verify that all beads have been resuspended, with no visible clumps in the suspension or bead pellets retained on the sides of the wells. Briefly spin to collect the liquid, being careful not to pellet the beads.

16 Incubate for 2 minutes at room temperature.

17 Put the plate or strip tube in the magnetic stand and leave until the solution is clear (up to 5 minutes).

18 Remove 50 µl of cleared supernatant to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the wells and store at 4°C overnight or at –20°C for prolonged storage.

3 Library Preparation

This chapter describes the steps to prepare cDNA NGS libraries for sequencing using the Illumina paired-read platform. For each sample to be sequenced, an individual dual-indexed and duplex-molecular-barcoded library is prepared.

Protocol steps in this section use the components listed in Table 13. Thaw and mix each component as directed in Table 13 before use (refer to the *Where Used* column). Remove the AMPure XP beads from cold storage and equilibrate to room temperature for at least 30 minutes in preparation for use on page 33. *Do not freeze the beads at any time.*

To process multiple samples, prepare reagent mixtures with overage at each step, without the cDNA library sample. Mixtures for preparation of 8 or 24 samples (including excess) are shown in tables as examples.

Table 13 Reagents thawed before use in protocol

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
Ligation Buffer (purple cap or bottle)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	page 29
T4 DNA Ligase (blue cap)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Place on ice just before use	Inversion	page 29
End Repair-A Tailing Buffer (yellow cap or bottle)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	page 31
End Repair-A Tailing Enzyme Mix (orange cap)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Place on ice just before use	Inversion	page 31
XT HS2 RNA Adaptor Oligo Mix (green cap)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	page 32

Step 1. Prepare the Ligation master mix

Prepare the Ligation master mix to allow equilibration to room temperature before use on page 32. Initiate this step before starting the End Repair/dA-tailing protocol; leave samples on ice while completing this step.

1 Vortex the thawed vial of Ligation Buffer for 15 seconds at high speed to ensure homogeneity.

CAUTION

The Ligation Buffer used in this step is viscous. Mix thoroughly by vortexing at high speed for 15 seconds before removing an aliquot for use. When combining with other reagents, mix well by pipetting up and down 15–20 times using a pipette set to at least 80% of the mixture volume or by vortexing at high speed for 10–20 seconds.

Use flat top vortex mixers when vortexing strip tubes or plates throughout the protocol. If reagents are mixed by vortexing, visually verify that adequate mixing is occurring.

2 Prepare the appropriate volume of Ligation master mix by combining the reagents in Table 14.

Slowly pipette the Ligation Buffer into a 1.5-ml tube, ensuring that the full volume is dispensed. Slowly add the T4 DNA Ligase, rinsing the enzyme tip with buffer solution after addition. Mix well by slowly pipetting up and down 15–20 times or seal the tube and vortex at high speed for 10–20 seconds. Spin briefly to collect the liquid.

Keep at room temperature for 30–45 minutes before use on page 32.

Table 14 Preparation of Ligation master mix

Reagent	Volume for 1 reaction	Volume for 8 reactions* (includes excess)	Volume for 24 reactions† (includes excess)
Ligation Buffer (purple cap or bottle)	23 µl	207 µl	575 µl
T4 DNA Ligase (blue cap)	2 µl	18 µl	50 µl
Total	**25 µl**	**225 µl**	**625 µl**

* The minimum supported run size for 16-reaction kits is 8 samples per run, with kits containing enough reagents for 2 runs of 8 samples each.

† The minimum supported run size for 96-reaction kits is 24 samples per run, with kits containing enough reagents for 4 runs of 24 samples each.

Step 2. Repair and dA-Tail the cDNA 3' ends

1 Preprogram a thermal cycler for the End Repair/dA-Tailing step with the program in Table 15. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 15 Thermal cycler program for End Repair/dA-Tailing*

Step	Temperature	Time
Step 1	20°C	15 minutes
Step 2	72°C	15 minutes
Step 3	4°C	Hold

* Use a reaction volume setting of 70 µl, if required for thermal cycler set up.

2 Vortex the thawed vial of End Repair-A Tailing Buffer for 15 seconds at high speed to ensure homogeneity. Visually inspect the solution; if any solids are observed, continue vortexing until all solids are dissolved.

CAUTION

The End Repair-A Tailing Buffer used in this step must be mixed thoroughly by vortexing at high speed for 15 seconds before removing an aliquot for use. When combining with other reagents, mix well either by pipetting up and down 15–20 times using a pipette set to at least 80% of the mixture volume or by vortexing at high speed for 5–10 seconds.

3 Prepare the appropriate volume of dA-Tailing master mix, by combining the reagents in Table 16.

Slowly pipette the End Repair-A Tailing Buffer into a 1.5-ml tube, ensuring that the full volume is dispensed. Slowly add the End Repair-A Tailing Enzyme Mix, rinsing the enzyme tip with buffer solution after addition. Mix well by pipetting up and down 15–20 times or seal the tube and vortex at high speed for 5–10 seconds. Spin briefly to collect the liquid and keep on ice.

Table 16 Preparation of End Repair/dA-Tailing master mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
End Repair-A Tailing Buffer (yellow cap or bottle)	16 µl	144 µl	400 µl
End Repair-A Tailing Enzyme Mix (orange cap)	4 µl	36 µl	100 µl
Total	**20 µl**	**180 µl**	**500 µl**

4 Add 20 µl of the End Repair/dA-Tailing master mix to each sample well containing approximately 50 µl of purified cDNA sample. Mix by pipetting up and down 15–20 times using a pipette set to 50 µl or cap the wells and vortex at high speed for 5–10 seconds.

5 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 15.

Step 3. Ligate the molecular-barcoded adaptor

1 Once the thermal cycler reaches the 4°C Hold step, transfer the samples to ice while setting up this step.

2 Preprogram a thermal cycler for the Ligation step with the program in Table 17. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 17 Thermal cycler program for Ligation*

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

* Use a reaction volume setting of 100 µl, if required for thermal cycler set up.

3 To each end-repaired/dA-tailed cDNA sample (approximately 70 µl), add 25 µl of the Ligation master mix that was prepared on page 29 and kept at room temperature. Mix by pipetting up and down at least 10 times using a pipette set to 70 µl or cap the wells and vortex at high speed for 5–10 seconds. Briefly spin the samples.

4 Add 5 µl of SureSelect XT HS2 RNA Adaptor Oligo Mix (green-capped tube) to each sample. Mix by pipetting up and down 15–20 times using a pipette set to 70 µl or cap the wells and vortex at high speed for 5–10 seconds.

NOTE Make sure to add the Ligation master mix and the RNA Adaptor Oligo Mix to the samples in separate addition steps as directed above, mixing after each addition.

5 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 17.

NOTE Unique molecular barcode sequences are incorporated into both ends of each library DNA fragment at this step.

Step 4. Purify the sample using AMPure XP beads

In this step, the adaptor-ligated cDNA libraries are purified using AMPure XP beads. Libraries prepared from <100 ng total RNA input or prepared from lower quality RNA samples (RIN 6–8) are subjected to an additional round of purification (see step 13 to step 20 below).

1. Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use.
2. Prepare fresh 70% ethanol for use in step 8. Prepare 400 µl per sample, plus excess, for libraries prepared from >100 ng RNA or 800 µl per sample, plus excess, for libraries prepared from <100 ng RNA.
3. Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Add 80 µl of homogeneous AMPure XP beads to each cDNA library sample (approximately 100 µl) in the PCR plate or strip tube. Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 to 10 minutes).
7. Keep the plate or strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 µl of freshly-prepared 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 to step 9 once.
11. Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol. Return the plate or strip tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
12. Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (typically 1–2 minutes).

13 Add the appropriate volume of nuclease-free water to each sample well, according to Table 18.

Table 18 Elution volume based on RNA input characteristics

Total RNA Input Quantity/Quality	Elution Volume	Rounds of Purification Required
≥100 ng **AND** RIN≥8	35 µl	1×
<100 ng **OR** RIN 6–8	50 µl	2×

14 Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate or strip tube to collect the liquid.

15 Incubate for 2 minutes at room temperature.

16 Put the plate or strip tube in the magnetic stand and leave for approximately 5 minutes, until the solution is clear.

17 Transfer the cleared supernatant (34 µl or 50 µl, see Table 19) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

Table 19 Supernatant transfer volume based on RNA input characteristics

Total RNA Input Quantity/Quality	Rounds of Purification Required	Volume of Supernatant Collected*
≥100 ng **AND** RIN≥8	1×	34 µl
<100 ng **OR** RIN 6–8	2×	50 µl

* For samples with single round of purification, avoid bead carryover by removing 34 µl of the 35 µl elution volume. Small amounts of bead carryover are acceptable for samples subjected to a second round of purification; collect the entire 50 µl of supernatant for these samples.

18 For samples prepared from ≥100 ng total RNA input with RIN≥8, proceed directly to "Step 5. Amplify the adaptor-ligated cDNA library" on page 36.

For lower-quantity or lower-quality input samples, complete step 19 and step 20 below.

For low input (<100 ng) or lower-quality (RIN 6–8) libraries only:

19 Add 60 µl of homogeneous AMPure XP beads to each 50-µl sample in the PCR plate or strip tube. Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.

20 Repeat the remaining purification steps, beginning with step 5 on page 33. For step 13, elute the re-purified cDNA library in 35 µl of nuclease-free water, then follow step 14 through step 17 as described.

After collecting 34 µl of re-purified supernatant in step 17, proceed directly to "Step 5. Amplify the adaptor-ligated cDNA library" on page 36.

Step 5. Amplify the adaptor-ligated cDNA library

This step uses the components listed in Table 20. Before you begin, thaw the reagents listed below and keep on ice.

Table 20 Reagents for PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Pipette up and down 15–20 times	page 38
5× Herculase II Buffer with dNTPs (clear cap)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	page 38
SureSelect XT HS2 Index Primer Pairs	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR),* –20°C	Vortexing	page 38

* Indexing primer pairs are provided in individual wells of strip tubes (16 reaction kits) or plates (96 reaction kits).

1 Determine the appropriate index pair assignment for each sample. See page 59–page 66 for sequences of the 8 bp index portion of the primers used to amplify the cDNA libraries in this step.

   Use a different indexing primer pair for each sample to be sequenced in the same lane.

CAUTION The SureSelect XT HS2 Index Primer Pairs are provided in single-use aliquots. To avoid cross-contamination of libraries, use each well in only one library preparation reaction. Do not retain and re-use any residual volume for subsequent experiments.

2 Preprogram a thermal cycler (with heated lid ON) with the program in Table 21. Immediately pause the program, and keep paused until samples are loaded in step 6.

Table 21 PCR Thermal Cycler Program*

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8–15 (See Table 22 for RNA input-based cycle number guidelines)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

* Use a reaction volume setting of 50 µl, if required for thermal cycler set up.

Table 22 PCR cycle number guidelines

Quantity of Input RNA	Cycle Number
1000 ng	8 cycles
250 ng	10 cycles
100 ng	11 cycles
50 ng	13 cycles
10 ng	15 cycles

CAUTION To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

3 Prepare the appropriate volume of PCR reaction mix, as described in Table 23, on ice. Mix well on a vortex mixer.

Table 23 Preparation of PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
5× Herculase II Buffer with dNTPs (clear cap)	10 µl	90 µl	250 µl
Herculase II Fusion DNA Polymerase (red cap)	1 µl	9 µl	25 µl
Total	**11 µl**	**99 µl**	**275 µl**

4 Add 11 µl of the PCR reaction mixture prepared in Table 23 to each purified DNA library sample (34 µl) in the PCR plate wells.

5 Add 5 µl of the appropriate SureSelect XT HS2 Index Primer Pair to each reaction.

Cap the wells then vortex at high speed for 5 seconds. Spin the plate or strip tube briefly to collect the liquid and release any bubbles.

6 Before adding the samples to the thermal cycler, resume the thermal cycling program in Table 21 to bring the temperature of the thermal block to 98°C. Once the cycler has reached 98°C in Segment 1 of the program, immediately place the sample plate or strip tube in the thermal block and close the lid.

CAUTION The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.

Step 6. Purify the amplified library with AMPure XP beads

1. Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use.
2. Prepare 400 µl of 70% ethanol per sample, plus excess, for use in step 8.
3. Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Add 50 µl of homogeneous AMPure XP beads to each 50-µl amplification reaction in the PCR plate or strip tube. Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 minutes).
7. Keep the plate or strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 µl of freshly-prepared 70% ethanol into each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 and step 9 step once.
11. Seal the wells with strip caps, then briefly spin the samples to collect the residual ethanol. Return the plate or strip tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
12. Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (typically 1–2 minutes).
13. Add 15 µl 1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA) to each sample well.
14. Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate or strip tube to collect the liquid.
15. Incubate for 2 minutes at room temperature.

16 Put the plate or strip tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

17 Remove the cleared supernatant (approximately 15 µl) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

NOTE It may not be possible to recover the entire 15-µl supernatant volume at this step; transfer the maximum possible amount of supernatant for further processing.

Step 7. Assess quality and quantity

Analyze each sample using one of the platforms listed in Table 24. Follow the instructions in the linked user guide provided for each assay in Table 24, after reviewing the SureSelect library qualification steps on page 42. Each analysis method provides an electropherogram showing the size distribution of fragments in the sample and tools for determining the concentration of DNA in the sample. See Table 25 for fragment size distribution guidelines. A representative electropherogram generated using the TapeStation system is provided to illustrate typical results.

Table 24 Library analysis options

Analysis platform	Assay used at this step	Link to assay instructions	Amount of library sample to analyze
Agilent 4200 or 4150 TapeStation system	D1000 ScreenTape	Agilent D1000 Assay Quick Guide	1 µl
Agilent 2100 Bioanalyzer system	DNA 1000 Kit	Agilent DNA 1000 Kit Guide	1 µl
Agilent 5200, 5300, or 5400 Fragment Analyzer system	NGS Fragment Kit (1-6000 bp)	Agilent NGS Fragment Kit (1-6000 bp) Kit Guide	2 µl

Table 25 Library qualification guidelines

Input RNA type	Expected library DNA fragment size peak position	NGS read lengths supported
High-quality RNA	200 to 700 bp	2 ×100 reads or 2 ×150 reads

Observation of a low molecular weight peak, in addition to the expected library fragment peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to that seen in the example electropherogram in this section. See *Troubleshooting* on page 71 for additional considerations.

1 Set up the instrument as instructed in the appropriate user guide (links provided in Table 24).

2 Prepare the samples for analysis and set up the assay as instructed in the appropriate user guide. Load the analysis assay into the instrument and complete the run.

3 Verify that the electropherogram shows the expected DNA fragment size peak position (see Table 25 for guidelines). A sample TapeStation system electropherogram is shown in Figure 2.

   Electropherograms obtained using the other analysis platform options listed in Table 24 are expected to show similar fragment size profiles.

4 Determine the concentration of the library DNA by integrating under the peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

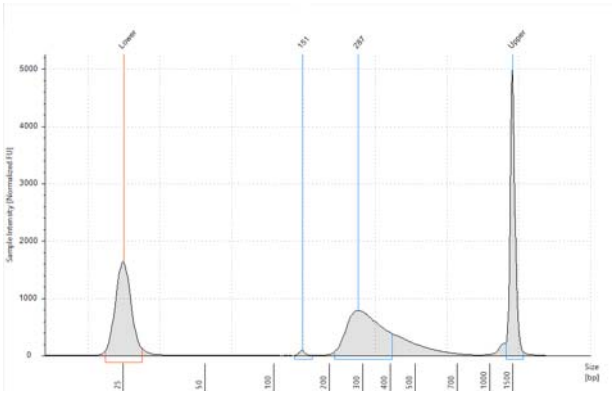


Figure 2 Final mRNA library analyzed using a D1000 ScreenTape.

Stopping Point If you do not continue to the next step, seal the sample wells and store at 4°C overnight or at –20°C for prolonged storage.

4 Guidelines for Multiplexed Sequencing

This chapter provides instructions to pool the indexed, molecular barcoded samples and provides guidelines for multiplexed sequencing.

Step 1. Pool samples for multiplexed sequencing

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample.

Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool using one of the following methods:

Method 1: Dilute each sample to be pooled to the same final concentration (typically 4 nM–15 nM, or the concentration of the most dilute sample) using Low TE, then combine equal volumes of all samples to create the final pool.

Method 2: Starting with samples at different concentrations, add the appropriate volume of each sample to achieve equimolar concentration in the pool, then adjust the pool to the desired final volume using Low TE. The formula below is provided for determination of the amount of each indexed sample to add to the pool.

$$\text{Volume of Index} = \frac{V(f) \times C(f)}{\# \times C(i)}$$

where ***V(f)*** is the final desired volume of the pool,

C(f) is the desired final concentration of all the DNA in the pool (typically 4 nM–15 nM or the concentration of the most dilute sample)

is the number of indexes, and

C(i) is the initial concentration of each indexed sample

Table 26 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µl at 10 nM DNA.

Table 26 Example of volume calculation for total volume of 20 µl at 10 nM concentration

Component	V(f)	C(i)	C(f)	#	Volume to use (µl)
Sample 1	20 µl	20 nM	10 nM	4	2.5
Sample 2	20 µl	10 nM	10 nM	4	5
Sample 3	20 µl	17 nM	10 nM	4	2.9
Sample 4	20 µl	25 nM	10 nM	4	2
Low TE					7.6

If you store the library before sequencing, add Tween 20 (Sigma-Aldrich p/n P9416) to 0.1% v/v and store at -20°C short term, or according to the instructions provided by your NGS service provider.

Step 2. Prepare sequencing samples

The final SureSelect XT HS2 mRNA library pool is ready for direct sequencing using standard Illumina paired-end primers and chemistry. Each fragment in the prepared library contains one mRNA-derived insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform, as shown in Figure 3.

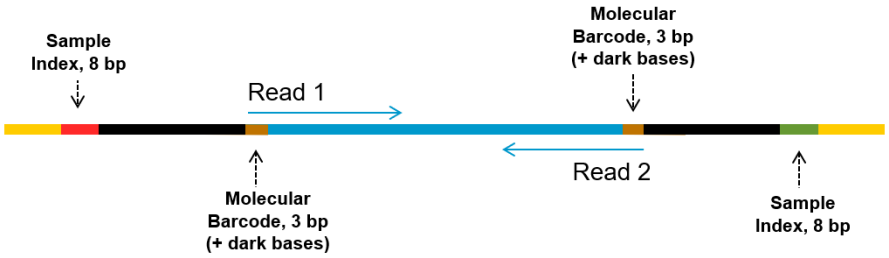


Figure 3 Content of SureSelect XT HS2 mRNA sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the dual sample indexes (red and green), dual molecular barcodes (brown) and the library bridge PCR primers (yellow).

Libraries can be sequenced on the Illumina HiSeq, MiSeq, NextSeq, or NovaSeq platform using the run type and chemistry combinations shown in Table 27.

The optimal seeding concentration for SureSelect XT HS2 mRNA libraries varies according to sequencing platform, run type, and Illumina kit version. See Table 27 for guidelines. Seeding concentration and cluster density may also need to be optimized based on the library DNA fragment size range and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in Table 27.

Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Table 27 Illumina Kit Configuration Selection Guidelines

Platform	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
HiSeq 2500	Rapid Run	2 × 100 bp	200 Cycle Kit	v2	9–10 pM
HiSeq 2500	High Output	2 × 100 bp	4×50 Cycle Kits*	v3	9–10 pM
HiSeq 2500	High Output	2 × 100 bp	250 Cycle Kit	v4	12–14 pM
MiSeq	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	9–10 pM
MiSeq	All Runs	2 × 75 bp	150 Cycle Kit	v3	12–16 pM
NextSeq 500/550	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2.5	1.2–1.5 pM
HiSeq 3000/4000	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1	230–240 pM
NovaSeq 6000	Standard Workflow Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1.0 or v1.5	300–600 pM
NovaSeq 6000	Xp Workflow Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1.0 or v1.5	200–400 pM

* A single 200-cycle kit does not include enough reagents to complete Reads 1 and 2 in addition to the 8-bp i7 and 8-bp i5 index reads in this format. If preferred, the additional reads may be supported by using one 200-cycle kit plus one 50-cycle kit.

Step 3. Do the sequencing run and analyze the data

The guidelines below provide an overview of SureSelect XT HS2 RNA library sequencing run setup and analysis considerations. Links are provided for additional details for various NGS platforms and analysis pipeline options.

- Each of the sample-level indexes requires an 8-bp index read. For complete index sequence information, see page 58 through page 69.
- For the HiSeq, NextSeq, and NovaSeq platforms, set up the run using the instrument's user interface, following the guidelines on page 49.
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed on page 49 to page 52 to generate a custom sample sheet.
- Demultiplex using Illumina's bcl2fastq software to generate paired end reads based on the dual indexes and remove sequences with incorrectly paired P5 and P7 indexes.
- Library fragments include a degenerate molecular barcode (MBC) in each strand (see Figure 3 on page 46). Note that unlike DNA, where both strands are present and the MBCs in the strands can be matched to form a duplex consensus read, single-stranded RNA stops at single consensus generation.
- The MBC sequence and dark bases are located at the 5' end of both Read 1 and Read 2. Use the Agilent Genomics NextGen Toolkit (AGeNT) for molecular barcode extraction and trimming (see page 53 for more information). If your sequence analysis pipeline excludes MBCs and is incompatible with AGeNT, you can trim or mask the first five bases from each read before alignment as described in the *Note* on page 53.
- Before aligning reads to reference sequences, Illumina adaptor sequences should be trimmed from the reads using Agilent's AGeNT trimmer module, which properly accounts for the degenerate MBCs in the adaptor sequence. See page 53 for more information. Do not use the adaptor trimming options in Illumina Experiment Manager (IEM). Make sure any IEM adaptor trimming option checkboxes are cleared (deselected) when setting up the sequencing run.

HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface, using the settings shown in Table 28. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface and enter the **Cycles** settings in Table 28.

For the NextSeq or NovaSeq platform, open the *Run Setup* screen of the instrument control software interface and enter the **Read Length** settings in Table 28. In the **Custom Primers** section, clear (do **not** select) the checkboxes for all primers (*Read 1, Read 2, Index 1* and *Index 2*).

Table 28 Run settings

Run Segment	Cycles/Read Length
Read 1	100 or 150
Index 1 (i7)	8
Index 2 (i5)	8
Read 2	100 or 150

MiSeq platform sequencing run setup guidelines

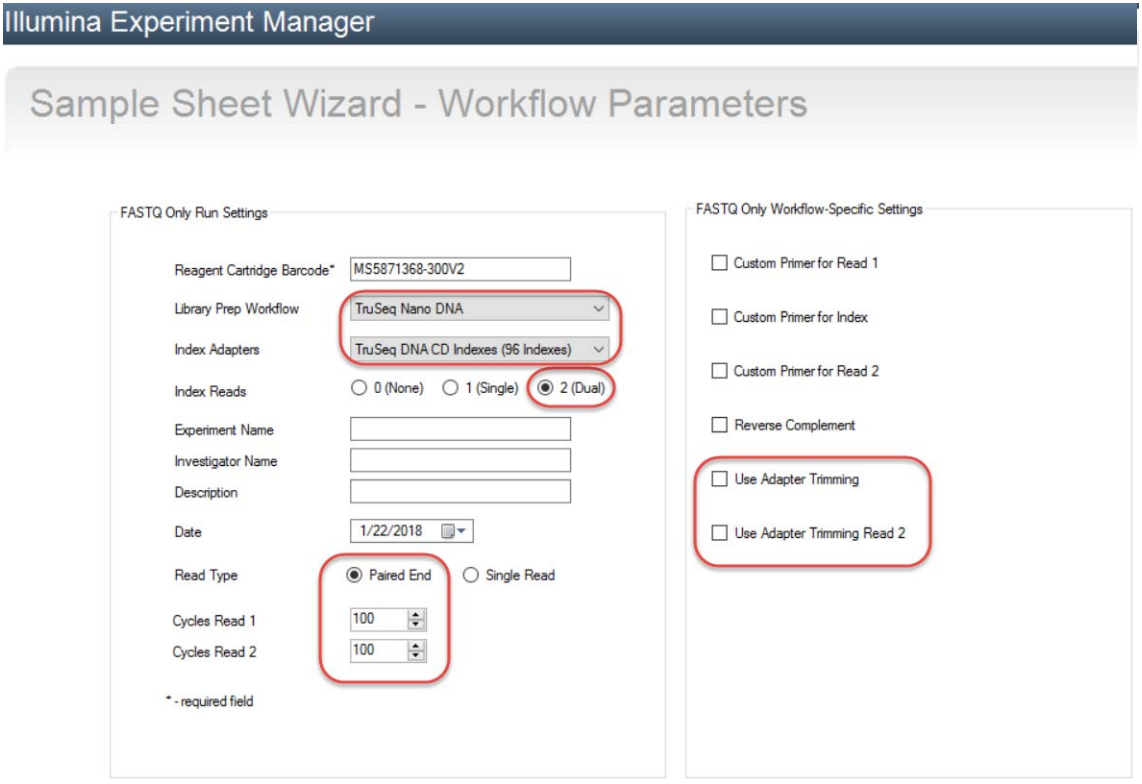
Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below. Once a Sample Sheet has been generated, index sequences need to be manually changed to the SureSelect XT HS2 indexes used for each sample. See Table 34 on page 59 though Table 41 on page 66 for nucleotide sequences of the SureSelect XT HS2 index pairs.

Set up a custom Sample Sheet:

1 In the IEM software, create a Sample Sheet for the MiSeq platform using the following Workflow selections.
   - Under **Category**, select *Other*.
   - Under **Application**, select *FASTQ Only*.

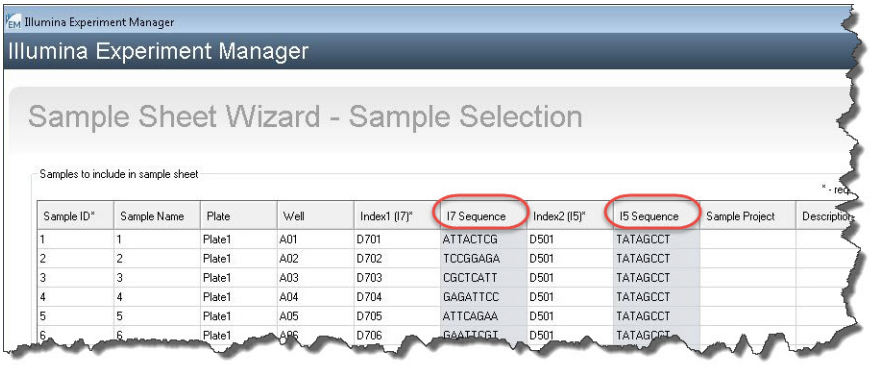
2 On the **Workflow Parameters** screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA**. In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. Make sure to clear both adaptor-trimming checkboxes under *FASTQ Only Workflow-Specific Settings* (circled below), since these are selected by default.

If **TruSeq Nano DNA** is not available in the *Sample Prep Kit* field, instead select **TruSeq HT**.



3 Using the **Sample Sheet Wizard**, set up a New Plate, entering the required information for each sample to be sequenced. In the **I7 Sequence** column, assign each sample to any of the Illumina i7 indexes. The index will be corrected to the i7 sequence from the SureSelect XT HS2 index pair at a later stage.

Likewise, in the **I5 Sequence** column, assign any of the Illumina i5 indexes, to be corrected to the i5 sequence from the SureSelect XT HS2 index pair at a later stage.

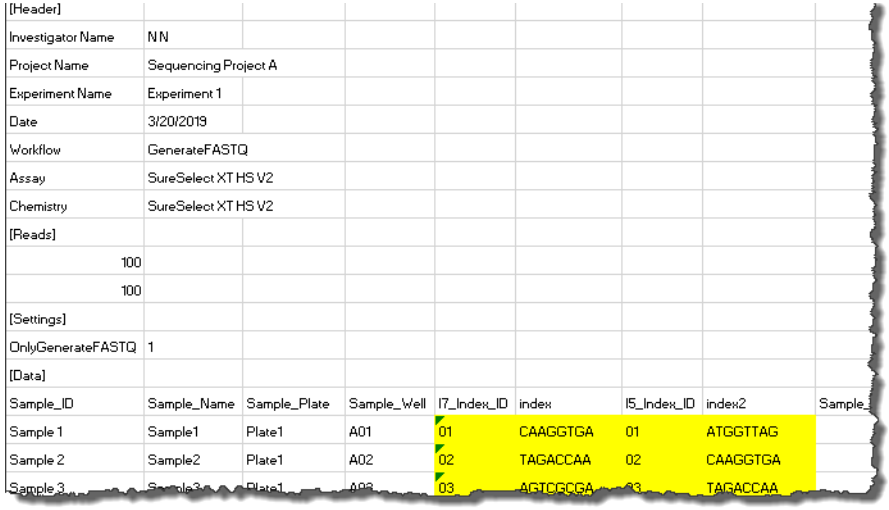


Sample ID*	Sample Name	Plate	Well	Index1 (I7)*	I7 Sequence	Index2 (I5)*	I5 Sequence	Sample Project	Description
1	1	Plate1	A01	D701	ATTACTCG	D501	TATAGCCT		
2	2	Plate1	A02	D702	TCCGGAGA	D501	TATAGCCT		
3	3	Plate1	A03	D703	CGCTCATT	D501	TATAGCCT		
4	4	Plate1	A04	D704	GAGATTCC	D501	TATAGCCT		
5	5	Plate1	A05	D705	ATTCAGAA	D501	TATAGCCT		
6	6	Plate1	A06	D706	GAATTCGT	D501	TATAGCCT		

4 Finish the sample sheet setup tasks and save the sample sheet file.

Editing the Sample Sheet to include SureSelect XT HS2 dual indexes

- Open the Sample Sheet file in a text editor and edit the i7 and i5 index information for each sample in columns 5–8 (highlighted below). See page 59–page 66 for nucleotide sequences of the SureSelect XT HS2 indexes.
- In column 5 under **I7_Index_ID**, enter the SureSelect XT HS2 index pair number assigned to the sample. In column 6 under **index**, enter the corresponding P7 index sequence.
- In column 7 under **I5_Index_ID**, enter the SureSelect XT HS2 index pair number assigned to the sample. In column 8 under **index2**, enter the corresponding P5 index sequence.



[Header]								
Investigator Name	NN							
Project Name	Sequencing Project A							
Experiment Name	Experiment 1							
Date	3/20/2019							
Workflow	GenerateFASTQ							
Assay	SureSelect XT HS V2							
Chemistry	SureSelect XT HS V2							
[Reads]								
100								
100								
[Settings]								
OnlyGenerateFASTQ	1							
[Data]								
Sample_ID	Sample_Name	Sample_Plate	Sample_Well	I7_Index_ID	index	I5_Index_ID	index2	Sample_
Sample 1	Sample1	Plate1	A01	01	CAAGGTGA	01	ATGGTTAG	
Sample 2	Sample2	Plate1	A02	02	TAGACCAA	02	CAAGGTGA	
Sample 3	Sample3	Plate1	A03	03	AGTCGCGA	03	TAGACCAA	

Figure 4 Sample sheet for SureSelect XT HS2 library sequencing

5 Save the edited Sample Sheet in an appropriate file location for use in the run.

Sequence analysis resources

Guidelines are provided below for typical NGS analysis pipeline steps appropriate for SureSelect XT HS2 RNA library data analysis. Your NGS analysis pipeline may vary.

Use the Illumina bcl2fastq software to generate paired end reads by demultiplexing sequences based on the dual indexes and to remove sequences with incorrectly paired P5 and P7 indexes.

The demultiplexed FASTQ data needs to be pre-processed to remove sequencing adaptors and extract the molecular barcode (MBC) sequences using the Agilent Genomics NextGen Toolkit (AGeNT). AGeNT is a set of Java-based software modules that provide MBC pre-processing adaptor trimming and duplicate read identification. This toolkit is designed to enable building, integrating, maintaining, and troubleshooting internal analysis pipelines for users with bioinformatics expertise. For additional information and to download this toolkit, visit the AGeNT page at www.agilent.com.

NOTE

If your sequence analysis pipeline excludes MBCs, you can remove the first 5 bases from Read 1 and Read 2 by either masking or trimming before proceeding to further analysis. To remove during demultiplexing via masking, include the base mask **N5Y*,I8,I8,N5Y*** (where * may be replaced with the actual read length, matching the read length value in the RunInfo.xml file). Alternatively, the first 5 bases may be trimmed from the demultiplexed fastq files using a suitable processing tool of your choice, such as seqtk. Alternatively, the AGeNT trimmer module can be used to remove the MBCs and properly remove adaptor sequences as well. Standard adaptor trimmers will fail to remove the MBC sequences from the opposite adaptor (refer to Figure 3).

The trimmed reads should be aligned, and MBC tags added to the aligned BAM file using a suitable tool such as the BWA-MEM. Once alignment and tagging are complete, the AGeNT LocatIt module may be used to generate consensus reads and mark or remove duplicates. The resulting BAM files are ready for downstream analysis including variant discovery.

Strandedness guidelines

The SureSelect XT HS2 mRNA sequencing library preparation method preserves RNA strandedness using dUTP second-strand marking. The sequence of read 1, which starts at the P5 end, matches the reverse complement of the poly-A RNA transcript strand. Read 2, which starts at the P7 end, matches the poly-A RNA transcript strand. When running analysis of this data to determine strandedness, it is important to include this information. For example, when using the Picard tools (https://broadinstitute.github.io/picard) to calculate RNA sequencing metrics, it is important to include the parameter *STRAND_SPECIFICITY*= *SECOND_READ_TRANSCRIPTION_STRAND* to correctly calculate the strand specificity metrics.

5 Reference

This chapter contains reference information, including component kit contents, index sequences, and troubleshooting information.

Kit Contents

SureSelect XT HS2 mRNA Reagent Kits include the component kits listed in Table 29. Detailed contents of each of the multi-part component kits listed in Table 29 are shown in Table 30 through Table 33 on the following pages.

Table 29 Component Kits

Component Kit Name	Storage Condition	Component Kit Part Number	
		16 Reaction Kits	96 Reaction Kits
Standard Component Modules			
SureSelect Poly-A Selection Module (Pre PCR)	+4°C	5190-6410	5190-6411
SureSelect cDNA Module (Pre PCR)	–20°C	5500-0148	5500-0149
SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0150	5500-0151
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	–20°C	5191-5687 (Index Pairs 1–16)	5191-5688 (Index Pairs 1–96), 5191-5689 (Index Pairs 97–192), 5191-5690 (Index Pairs 193–288), OR 5191-5691 (Index Pairs 289–384)
Optional Component Modules			
SureSelect RNA AMPure® XP Beads	+4°C	5191-6670*	5191-6671†

* Provided only with 16-Reaction Reagent Kit part number G9996A.

† Provided only with 96-Reaction Reagent Kit part numbers G9998A, G9998B, G9998C, G9998D.

Table 30 SureSelect Poly-A Selection Module (Pre PCR) Content

Kit Component	16 Reaction Kit Format	96 Reaction Kit Format
Oligo(dT) Microparticles	tube with brown cap	bottle
Bead Binding Buffer	tube with purple cap	bottle
Bead Washing Buffer	bottle	bottle
Bead Elution Buffer	tube with green cap	bottle

Table 31 SureSelect cDNA Module (Pre PCR) Content

Kit Component	16 Reaction Kit Format	96 Reaction Kit Format
2X Priming Buffer	tube with purple cap	tube with purple cap
First Strand Master Mix*	amber tube with amber cap	amber tube with amber cap
Second Strand Enzyme Mix	tube with blue cap	bottle
Second Strand Oligo Mix	tube with yellow cap	tube with yellow cap

* The First Strand Master Mix contains actinomycin-D. Keep the reagent in the supplied amber vial to protect the contents from exposure to light.

Table 32 SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR) Content

Kit Component	16 Reaction Kit Format	96 Reaction Kit Format
End Repair-A Tailing Enzyme Mix	tube with orange cap	tube with orange cap
End Repair-A Tailing Buffer	tube with yellow cap	bottle
T4 DNA Ligase	tube with blue cap	tube with blue cap
Ligation Buffer	tube with purple cap	bottle
XT HS2 RNA Adaptor Oligo Mix	tube with green cap	tube with green cap
Herculase II Fusion DNA Polymerase	tube with red cap	tube with red cap
5× Herculase II Reaction Buffer with dNTPs	tube with clear cap	tube with clear cap

Table 33 SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) Content

Kit Component	16 Reaction Kit Format	96 Reaction Kit Format
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	Blue 8-well strip tube (index pairs 1-8), AND White 8-well strip tube (index pairs 9-16)	Orange 96-well plate (index pairs 1–96), OR Blue 96-well plate (index pairs 97–192), OR Green 96-well plate (index pairs 193–288), OR Red 96-well plate (index pairs 289–384)

SureSelect XT HS2 Index Primer Pair Information

The SureSelect XT HS2 Index Primer Pairs are provided pre-combined. Each member of the primer pair contains a unique 8-bp P5 or P7 index, resulting in dual-indexed NGS libraries. The nucleotide sequence of the index portion of each primer is provided in Table 34 through Table 41. See page 72 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

NOTE

P7 indexes are shown in a single orientation, applicable to any of the supported Illumina platforms. P5 indexes are shown in two orientations for different platforms; check the table column headings carefully before selecting the P5 sequences.

The first P5 index orientation is applicable to the supported platforms NovaSeq 6000 with v1.0 chemistry, MiSeq, and HiSeq 2500. This orientation is also applicable to the HiSeq 2000 platform that is not specifically supported in this user manual.

The second P5 index orientation is applicable to the supported platforms NovaSeq 6000 with v1.5 chemistry, NextSeq 500/550, HiSeq 4000 and HiSeq 3000. This orientation is also applicable to the iSeq 100, MiniSeq, and HiSeq X platforms that are not specifically supported in this user manual.

One primer pair is provided in each well of 8-well strip tubes (16 reaction kits; see Figure 5 for a map) or of 96-well plates (96 reaction kits; see page 68 through page 69 for plate maps). Each well contains a single-use aliquot of a specific pair of forward plus reverse primers.

Table 34 SureSelect XT HS2 Index Primer Pairs 1–48, provided in orange 96-well plate or in strip tubes

Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000	Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000
1	A01	CAAGGTGA	ATGGTTAG	CTAACCAT	25	A04	AGATGGAT	TGGCACCA	TGGTGCCA
2	B01	TAGACCAA	CAAGGTGA	TCACCTTG	26	B04	GAATTGTG	AGATGGAT	ATCCATCT
3	C01	AGTCGCGA	TAGACCAA	TTGGTCTA	27	C04	GAGCACTG	GAATTGTG	CACAATTC
4	D01	CGGTAGAG	AGTCGCGA	TCGCGACT	28	D04	GTTGCGGA	GAGCACTG	CAGTGCTC
5	E01	TCAGCATC	AAGGAGCG	CGCTCCTT	29	E04	AATGGAAC	GTTGCGGA	TCCGCAAC
6	F01	AGAAGCAA	TCAGCATC	GATGCTGA	30	F04	TCAGAGGT	AATGGAAC	GTTCCATT
7	G01	GCAGGTTC	AGAAGCAA	TTGCTTCT	31	G04	GCAACAAT	TCAGAGGT	ACCTCTGA
8	H01	AAGTGTCT	GCAGGTTC	GAACCTGC	32	H04	GTCGATCG	GCAACAAT	ATTGTTGC
9	A02	CTACCGAA	AAGTGTCT	AGACACTT	33	A05	ATGGTAGC	GTCGATCG	CGATCGAC
10	B02	TAGAGCTC	CTACCGAA	TTCGGTAG	34	B05	CGCCAATT	ATGGTAGC	GCTACCAT
11	C02	ATGTCAAG	TAGAGCTC	GAGCTCTA	35	C05	GACAATTG	CGCCAATT	AATTGGCG
12	D02	GCATCATA	ATGTCAAG	CTTGACAT	36	D05	ATATTCCG	GACAATTG	CAATTGTC
13	E02	GACTTGAC	GCATCATA	TATGATGC	37	E05	TCTACCTC	ATATTCCG	CGGAATAT
14	F02	CTACAATG	GACTTGAC	GTCAAGTC	38	F05	TCGTCGTG	TCTACCTC	GAGGTAGA
15	G02	TCTCAGCA	CTACAATG	CATTGTAG	39	G05	ATGAGAAC	TCGTCGTG	CACGACGA
16	H02	AGACACAC	TCTCAGCA	TGCTGAGA	40	H05	GTCCTATA	ATGAGAAC	GTTCTCAT
17	A03	CAGGTCTG	AGACACAC	GTGTGTCT	41	A06	AATGACCA	GTCCTATA	TATAGGAC
18	B03	AATACGCG	CAGGTCTG	CAGACCTG	42	B06	CAGACGCT	AATGACCA	TGGTCATT
19	C03	GCACACAT	AATACGCG	CGCGTATT	43	C06	TCGAACTG	CAGACGCT	AGCGTCTG
20	D03	CTTGCATA	GCACACAT	ATGTGTGC	44	D06	CGCTTCCA	TCGAACTG	CAGTTCGA
21	E03	ATCCTCTT	CTTGCATA	TATGCAAG	45	E06	TATTCCTG	CGCTTCCA	TGGAAGCG
22	F03	GCACCTAA	ATCCTCTT	AAGAGGAT	46	F06	CAAGTTAC	TATTCCTG	CAGGAATA
23	G03	TGCTGCTC	GCACCTAA	TTAGGTGC	47	G06	CAGAGCAG	CAAGTTAC	GTAACTTG
24	H03	TGGCACCA	TGCTGCTC	GAGCAGCA	48	H06	CGCGCAAT	CAGAGCAG	CTGCTCTG

Table 35 SureSelect XT HS2 Index Primer Pairs 49–96, provided in orange 96-well plate

Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000	Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000
49	A07	TGAGGAGT	CGCGCAAT	ATTGCGCG	73	A10	AACGCATT	ATAGTGAC	GTCACTAT
50	B07	ATGACGAA	TGAGGAGT	ACTCCTCA	74	B10	CAGTTGCG	AACGCATT	AATGCGTT
51	C07	TACGGCGA	ATGACGAA	TTCGTCAT	75	C10	TGCCTCGA	CAGTTGCG	CGCAACTG
52	D07	AGCGAGTT	TACGGCGA	TCGCCGTA	76	D10	AAGGCTTA	TGCCTCGA	TCGAGGCA
53	E07	TGTATCAC	AGCGAGTT	AACTCGCT	77	E10	GCAATGAA	AAGGCTTA	TAAGCCTT
54	F07	GATCGCCT	TGTATCAC	GTGATACA	78	F10	AAGAACCT	GCAATGAA	TTCATTGC
55	G07	GACTCAAT	GATCGCCT	AGGCGATC	79	G10	CTGTGCCT	AAGAACCT	AGGTTCTT
56	H07	CAGCTTGC	GACTCAAT	ATTGAGTC	80	H10	TACGTAGC	CTGTGCCT	AGGCACAG
57	A08	AGCTGAAG	CAGCTTGC	GCAAGCTG	81	A11	AAGTGGAC	TACGTAGC	GCTACGTA
58	B08	ATTCCGTG	AGCTGAAG	CTTCAGCT	82	B11	CAACCGTG	AAGTGGAC	GTCCACTT
59	C08	TATGCCGC	ATTCCGTG	CACGGAAT	83	C11	CTGTTGTT	CAACCGTG	CACGGTTG
60	D08	TCAGCTCA	TATGCCGC	GCGGCATA	84	D11	GCACGATG	CTGTTGTT	AACAACAG
61	E08	AACTGCAA	TCAGCTCA	TGAGCTGA	85	E11	GTACGGAC	GCACGATG	CATCGTGC
62	F08	ATTAGGAG	AACTGCAA	TTGCAGTT	86	F11	CTCCAAGC	GTACGGAC	GTCCGTAC
63	G08	CAGCAATA	ATTAGGAG	CTCCTAAT	87	G11	TAGTCTGA	CTCCAAGC	GCTTGGAG
64	H08	GCCAAGCT	CAGCAATA	TATTGCTG	88	H11	TTCGCCGT	TAGTCTGA	TCAGACTA
65	A09	TCCGTTAA	GCCAAGCT	AGCTTGGC	89	A12	GAACTAAG	ATACGAAG	CTTCGTAT
66	B09	GTGCAACG	TCCGTTAA	TTAACGGA	90	B12	AAGCCATC	GAGATTCA	TGAATCTC
67	C09	AGTAACGC	GTGCAACG	CGTTGCAC	91	C12	AACTCTTG	AAGCCATC	GATGGCTT
68	D09	CATAGCCA	AGTAACGC	GCGTTACT	92	D12	GTAGTCAT	AACTCTTG	CAAGAGTT
69	E09	CACTAGTA	CATAGCCA	TGGCTATG	93	E12	CTCGCTAG	GTAGTCAT	ATGACTAC
70	F09	TTAGTGCG	CACTAGTA	TACTAGTG	94	F12	AGTCTTCA	CAGTATCA	TGATACTG
71	G09	TCGATACA	TTAGTGCG	CGCACTAA	95	G12	TCAAGCTA	CTTCGTAC	GTACGAAG
72	H09	ATAGTGAC	TCGATACA	TGTATCGA	96	H12	CTTATCCT	TCAAGCTA	TAGCTTGA

Table 36 SureSelect XT HS2 Index Primer Pairs 97–144, provided in blue 96-well plate

Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000	Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000
97	A01	TCATCCTT	CTTATCCT	AGGATAAG	121	A04	CAGGCAGA	AGACGCCT	AGGCGTCT
98	B01	AACACTCT	TCATCCTT	AAGGATGA	122	B04	TCCGCGAT	CAGGCAGA	TCTGCCTG
99	C01	CACCTAGA	AACACTCT	AGAGTGTT	123	C04	CTCGTACG	TCCGCGAT	ATCGCGGA
100	D01	AGTTCATG	CACCTAGA	TCTAGGTG	124	D04	CACACATA	CTCGTACG	CGTACGAG
101	E01	GTTGGTGT	AGTTCATG	CATGAACT	125	E04	CGTCAAGA	CACACATA	TATGTGTG
102	F01	GCTACGCA	GTTGGTGT	ACACCAAC	126	F04	TTCGCGCA	CGTCAAGA	TCTTGACG
103	G01	TCAACTGC	GCTACGCA	TGCGTAGC	127	G04	CGACTACG	TTCGCGCA	TGCGCGAA
104	H01	AAGCGAAT	TCAACTGC	GCAGTTGA	128	H04	GAAGGTAT	CGACTACG	CGTAGTCG
105	A02	GTGTTACA	AAGCGAAT	ATTCGCTT	129	A05	TTGGCATG	GAAGGTAT	ATACCTTC
106	B02	CAAGCCAT	GTGTTACA	TGTAACAC	130	B05	CGAATTCA	TTGGCATG	CATGCCAA
107	C02	CTCTCGTG	CAAGCCAT	ATGGCTTG	131	C05	TTAGTTGC	CGAATTCA	TGAATTCG
108	D02	TCGACAAC	CTCTCGTG	CACGAGAG	132	D05	GATGCCAA	TTAGTTGC	GCAACTAA
109	E02	TCGATGTT	TCGACAAC	GTTGTCGA	133	E05	AGTTGCCG	GATGCCAA	TTGGCATC
110	F02	CAAGGAAG	TCGATGTT	AACATCGA	134	F05	GTCCACCT	AGTTGCCG	CGGCAACT
111	G02	ATTGATGC	AGAGAATC	GATTCTCT	135	G05	ATCAAGGT	GTCCACCT	AGGTGGAC
112	H02	TCGCAGAT	TTGATGGC	GCCATCAA	136	H05	GAACCAGA	ATCAAGGT	ACCTTGAT
113	A03	GCAGAGAC	TCGCAGAT	ATCTGCGA	137	A06	CATGTTCT	GAACCAGA	TCTGGTTC
114	B03	CTGCGAGA	GCAGAGAC	GTCTCTGC	138	B06	TCACTGTG	CATGTTCT	AGAACATG
115	C03	CAACCAAC	CTGCGAGA	TCTCGCAG	139	C06	ATTGAGCT	TCACTGTG	CACAGTGA
116	D03	ATCATGCG	CAACCAAC	GTTGGTTG	140	D06	GATAGAGA	ATTGAGCT	AGCTCAAT
117	E03	TCTGAGTC	ATCATGCG	CGCATGAT	141	E06	TCTAGAGC	GATAGAGA	TCTCTATC
118	F03	TCGCCTGT	TCTGAGTC	GACTCAGA	142	F06	GAATCGCA	TCTAGAGC	GCTCTAGA
119	G03	GCGCAATT	TCGCCTGT	ACAGGCGA	143	G06	CTTCACGT	GAATCGCA	TGCGATTC
120	H03	AGACGCCT	GCGCAATT	AATTGCGC	144	H06	CTCCGGTT	CTTCACGT	ACGTGAAG

Table 37 SureSelect XT HS2 Index Primer Pairs 145–192, provided in blue 96-well plate

Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000	Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000
145	A07	TGTGACTA	CTCCGGTT	AACCGGAG	169	A10	CGCTCAGA	CTAACAAG	CTTGTTAG
146	B07	GCTTCCAG	TGTGACTA	TAGTCACA	170	B10	TAACGACA	CGCTCAGA	TCTGAGCG
147	C07	CATCCTGT	GCTTCCAG	CTGGAAGC	171	C10	CATACTTG	TAACGACA	TGTCGTTA
148	D07	GTAATACG	CATCCTGT	ACAGGATG	172	D10	AGATACGA	CATACTTG	CAAGTATG
149	E07	GCCAACAA	GTAATACG	CGTATTAC	173	E10	AATCCGAC	AGATACGA	TCGTATCT
150	F07	CATGACAC	GCCAACAA	TTGTTGGC	174	F10	TGAAGTAC	AATCCGAC	GTCGGATT
151	G07	TGCAATGC	CATGACAC	GTGTCATG	175	G10	CGAATCAT	TGAAGTAC	GTACTTCA
152	H07	CACATTCG	TGCAATGC	GCATTGCA	176	H10	TGATTGGC	CGAATCAT	ATGATTCG
153	A08	CAATCCGA	CACATTCG	CGAATGTG	177	A11	TCGAAGGA	TGATTGGC	GCCAATCA
154	B08	CATCGACG	CAATCCGA	TCGGATTG	178	B11	CAGTCATT	TCGAAGGA	TCCTTCGA
155	C08	GTGCGCTT	CATCGACG	CGTCGATG	179	C11	CGCGAACA	CAGTCATT	AATGACTG
156	D08	ATAGCGTT	GTGCGCTT	AAGCGCAC	180	D11	TACGGTTG	CGCGAACA	TGTTCGCG
157	E08	GAGTAAGA	ATAGCGTT	AACGCTAT	181	E11	AGAACCGT	TACGGTTG	CAACCGTA
158	F08	CTGACACA	GAGTAAGA	TCTTACTC	182	F11	AGGTGCTT	AGAACCGT	ACGGTTCT
159	G08	ATACGTGT	CTGACACA	TGTGTCAG	183	G11	ATCGCAAC	AGGTGCTT	AAGCACCT
160	H08	GACCGAGT	ATACGTGT	ACACGTAT	184	H11	GCCTCTCA	ATCGCAAC	GTTGCGAT
161	A09	GCAGTTAG	GACCGAGT	ACTCGGTC	185	A12	TCGCGTCA	GCCTCTCA	TGAGAGGC
162	B09	CGTTCGTC	GCAGTTAG	CTAACTGC	186	B12	GAGTGCGT	TCGCGTCA	TGACGCGA
163	C09	CGTTAACG	CGTTCGTC	GACGAACG	187	C12	CGAACACT	GCATAAGT	ACTTATGC
164	D09	TCGAGCAT	CGTTAACG	CGTTAACG	188	D12	TAAGAGTG	AGAAGACG	CGTCTTCT
165	E09	GCCGTAAC	TCGAGCAT	ATGCTCGA	189	E12	TGGATTGA	TAAGAGTG	CACTCTTA
166	F09	GAGCTGTA	GCCGTAAC	GTTACGGC	190	F12	AGGACATA	TGGATTGA	TCAATCCA
167	G09	AGGAAGAT	GAGCTGTA	TACAGCTC	191	G12	GACATCCT	AGGACATA	TATGTCCT
168	H09	CTAACAAG	AGGAAGAT	ATCTTCCT	192	H12	GAAGCCTC	GACATCCT	AGGATGTC

Table 38 SureSelect XT HS2 Index Primer Pairs 193–240, provided in green 96-well plate

Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000	Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000
193	A01	GTCTCTTC	GAAGCCTC	GAGGCTTC	217	A04	GCGGTATG	CACGAGCT	AGCTCGTG
194	B01	AGTCACTT	GTCTCTTC	GAAGAGAC	218	B04	TCTATGCG	GCGGTATG	CATACCGC
195	C01	AGCATACA	AGTCACTT	AAGTGACT	219	C04	AGGTGAGA	TCTATGCG	CGCATAGA
196	D01	TCAGACAA	AGCATACA	TGTATGCT	220	D04	CACAACTT	AGGTGAGA	TCTCACCT
197	E01	TTGGAGAA	TCAGACAA	TTGTCTGA	221	E04	TTGTGTAC	CACAACTT	AAGTTGTG
198	F01	TTAACGTG	TTGGAGAA	TTCTCCAA	222	F04	TCACAAGA	TTGTGTAC	GTACACAA
199	G01	CGTCTGTG	TTAACGTG	CACGTTAA	223	G04	GAAGACCT	TCACAAGA	TCTTGTGA
200	H01	AACCTAAC	CGTCTGTG	CACAGACG	224	H04	AGTTCTGT	GAAGACCT	AGGTCTTC
201	A02	AGAGTGCT	AACCTAAC	GTTAGGTT	225	A05	GCAGTGTT	AGTTCTGT	ACAGAACT
202	B02	TTATCTCG	AGAGTGCT	AGCACTCT	226	B05	AGGCATGC	GCAGTGTT	AACACTGC
203	C02	CATCAGTC	TTATCTCG	CGAGATAA	227	C05	AAGGTACT	AGGCATGC	GCATGCCT
204	D02	AAGCACAA	CATCAGTC	GACTGATG	228	D05	CACTAAGT	AAGGTACT	AGTACCTT
205	E02	CAGTGAGC	AAGCACAA	TTGTGCTT	229	E05	GAGTCCTA	CACTAAGT	ACTTAGTG
206	F02	GTCGAAGT	CAGTGAGC	GCTCACTG	230	F05	AGTCCTTC	GAGTCCTA	TAGGACTC
207	G02	TCTCATGC	GTCGAAGT	ACTTCGAC	231	G05	TTAGGAAC	AGTCCTTC	GAAGGACT
208	H02	CAGAAGAA	TCTCATGC	GCATGAGA	232	H05	AAGTCCAT	TTAGGAAC	GTTCCTAA
209	A03	CGGATAGT	CAGAAGAA	TTCTTCTG	233	A06	GAATACGC	AAGTCCAT	ATGGACTT
210	B03	CACGTGAG	CGGATAGT	ACTATCCG	234	B06	TCCAATCA	GAATACGC	GCGTATTC
211	C03	TACGATAC	CACGTGAG	CTCACGTG	235	C06	CGACGGTA	TCCAATCA	TGATTGGA
212	D03	CGCATGCT	TACGATAC	GTATCGTA	236	D06	CATTGCAT	CGACGGTA	TACCGTCG
213	E03	GCTTGCTA	CGCATGCT	AGCATGCG	237	E06	ATCTGCGT	CATTGCAT	ATGCAATG
214	F03	GAACGCAA	GCTTGCTA	TAGCAAGC	238	F06	GTACCTTG	ATCTGCGT	ACGCAGAT
215	G03	ATCTACCA	GAACGCAA	TTGCGTTC	239	G06	GAGCATAC	GTACCTTG	CAAGGTAC
216	H03	CACGAGCT	ATCTACCA	TGGTAGAT	240	H06	TGCTTACG	GAGCATAC	GTATGCTC

Table 39 SureSelect XT HS2 Index Primer Pairs 241–288, provided in green 96-well plate

Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000	Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000
241	A07	AAGAGACA	TGCTTACG	CGTAAGCA	265	A10	CAATGCTG	CATGAATG	CATTCATG
242	B07	TAGCTATG	AAGAGACA	TGTCTCTT	266	B10	CTTGATCA	CAATGCTG	CAGCATTG
243	C07	TCTGCTAC	TAGCTATG	CATAGCTA	267	C10	GCGAATTA	CTTGATCA	TGATCAAG
244	D07	GTCACAGA	TCTGCTAC	GTAGCAGA	268	D10	GTTCGAGC	GCGAATTA	TAATTCGC
245	E07	CGATTGAA	GTCACAGA	TCTGTGAC	269	E10	GCCAGTAG	GTTCGAGC	GCTCGAAC
246	F07	GAGAGATT	CGATTGAA	TTCAATCG	270	F10	AAGGTCGA	GCCAGTAG	CTACTGGC
247	G07	TCATACCG	GAGAGATT	AATCTCTC	271	G10	AGTGAAGT	CACTTATG	CATAAGTG
248	H07	TCCGAACT	TCATACCG	CGGTATGA	272	H10	GTTGCAAG	ATAACGGC	GCCGTTAT
249	A08	AGAGAGAA	TCCGAACT	AGTTCGGA	273	A11	AGCCGGAA	GTTGCAAG	CTTGCAAC
250	B08	GATCGTTA	AGAGAGAA	TTCTCTCT	274	B11	AACAGCCG	AGCCGGAA	TTCCGGCT
251	C08	GCGCTAGA	GATCGTTA	TAACGATC	275	C11	CTAGTGTA	AACAGCCG	CGGCTGTT
252	D08	ATGACTCG	GCGCTAGA	TCTAGCGC	276	D11	GAGGCTCT	CTAGTGTA	TACACTAG
253	E08	CAATAGAC	ATGACTCG	CGAGTCAT	277	E11	CTCCGCAA	GAGGCTCT	AGAGCCTC
254	F08	CGATATGC	CAATAGAC	GTCTATTG	278	F11	CGCTATTG	CTCCGCAA	TTGCGGAG
255	G08	GTCAGAAT	CGATATGC	GCATATCG	279	G11	GTGTTGAG	CGCTATTG	CAATAGCG
256	H08	CATAAGGT	GCACTACT	AGTAGTGC	280	H11	TCACCGAC	GTGTTGAG	CTCAACAC
257	A09	TGTTGGTT	GATTCGGC	GCCGAATC	281	A12	CGGTAATC	TCACCGAC	GTCGGTGA
258	B09	ATACTCGC	TGTTGGTT	AACCAACA	282	B12	GTGACTGC	CGGTAATC	GATTACCG
259	C09	AATGCTAG	ATACTCGC	GCGAGTAT	283	C12	CGACTTGT	GTGACTGC	GCAGTCAC
260	D09	GCCTAGGA	AATGCTAG	CTAGCATT	284	D12	GATAGGAC	CGACTTGT	ACAAGTCG
261	E09	GCAACCGA	GCCTAGGA	TCCTAGGC	285	E12	AAGTACTC	GATAGGAC	GTCCTATC
262	F09	ATACTGCA	GCAACCGA	TCGGTTGC	286	F12	GCTCTCTC	AAGTACTC	GAGTACTT
263	G09	TCTCCTTG	ATACTGCA	TGCAGTAT	287	G12	CTACCAGT	GCTCTCTC	GAGAGAGC
264	H09	CATGAATG	TCTCCTTG	CAAGGAGA	288	H12	GATGAGAT	CTACCAGT	ACTGGTAG

Table 40 SureSelect XT HS2 Index Primer Pairs 289–336, provided in red 96-well plate

Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000	Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000
289	A01	AGATAGTG	GATGAGAT	ATCTCATC	313	A04	AGCTACAT	GATCCATG	CATGGATC
290	B01	AGAGGTTA	AGATAGTG	CACTATCT	314	B04	CGCTGTAA	AGCTACAT	ATGTAGCT
291	C01	CTGACCGT	AGAGGTTA	TAACCTCT	315	C04	CACTACCG	CGCTGTAA	TTACAGCG
292	D01	GCATGGAG	CTGACCGT	ACGGTCAG	316	D04	GCTCACGA	CACTACCG	CGGTAGTG
293	E01	CTGCCTTA	GCATGGAG	CTCCATGC	317	E04	TGGCTTAG	GCTCACGA	TCGTGAGC
294	F01	GCGTCACT	CTGCCTTA	TAAGGCAG	318	F04	TCCAGACG	TGGCTTAG	CTAAGCCA
295	G01	GCGATTAC	GCGTCACT	AGTGACGC	319	G04	AGTGGCAT	TCCAGACG	CGTCTGGA
296	H01	TCACCACG	GCGATTAC	GTAATCGC	320	H04	TGTACCGA	AGTGGCAT	ATGCCACT
297	A02	AGACCTGA	TCACCACG	CGTGGTGA	321	A05	AAGACTAC	TGTACCGA	TCGGTACA
298	B02	GCCGATAT	AGACCTGA	TCAGGTCT	322	B05	TGCCGTTA	AAGACTAC	GTAGTCTT
299	C02	CTTATTGC	GCCGATAT	ATATCGGC	323	C05	TTGGATCT	TGCCGTTA	TAACGGCA
300	D02	CGATACCT	CTTATTGC	GCAATAAG	324	D05	TCCTCCAA	TTGGATCT	AGATCCAA
301	E02	CTCGACAT	CGATACCT	AGGTATCG	325	E05	CGAGTCGA	TCCTCCAA	TTGGAGGA
302	F02	GAGATCGC	CTCGACAT	ATGTCGAG	326	F05	AGGCTCAT	CGAGTCGA	TCGACTCG
303	G02	CGGTCTCT	GAGATCGC	GCGATCTC	327	G05	GACGTGCA	AGGCTCAT	ATGAGCCT
304	H02	TAACTCAC	CGGTCTCT	AGAGACCG	328	H05	GAACATGT	GACGTGCA	TGCACGTC
305	A03	CACAATGA	TAACTCAC	GTGAGTTA	329	A06	AATTGGCA	GAACATGT	ACATGTTC
306	B03	GACTGACG	CACAATGA	TCATTGTG	330	B06	TGGAGACT	AATTGGCA	TGCCAATT
307	C03	CTTAAGAC	GACTGACG	CGTCAGTC	331	C06	AACTCACA	TGGAGACT	AGTCTCCA
308	D03	GAGTGTAG	CTTAAGAC	GTCTTAAG	332	D06	GTAGACTG	AACTCACA	TGTGAGTT
309	E03	TGCACATC	GAGTGTAG	CTACACTC	333	E06	CGTAGTTA	GTAGACTG	CAGTCTAC
310	F03	CGATGTCG	TGCACATC	GATGTGCA	334	F06	CGTCAGAT	CGTAGTTA	TAACTACG
311	G03	AACACCGA	CGATGTCG	CGACATCG	335	G06	AACGGTCA	CGTCAGAT	ATCTGACG
312	H03	GATCCATG	AACACCGA	TCGGTGTT	336	H06	GCCTTCAT	AACGGTCA	TGACCGTT

Table 41 SureSelect XT HS2 Index Primer Pairs 337–384, provided in red 96-well plate

Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000	Primer Pair #	Well	P7 Index	P5 Index NovaSeq (v1.0 chemistry), MiSeq, HiSeq 2500	P5 Index NovaSeq (v1.5 chemistry), NextSeq, HiSeq 4000, HiSeq 3000
337	A07	TGAGACGC	GCCTTCAT	ATGAAGGC	361	A10	CTGAGCTA	GCACAGTA	TACTGTGC
338	B07	CATCGGAA	TGAGACGC	GCGTCTCA	362	B10	CTTGCGAT	CTGAGCTA	TAGCTCAG
339	C07	TAGGACAT	CATCGGAA	TTCCGATG	363	C10	GAAGTAGT	CTTGCGAT	ATCGCAAG
340	D07	AACACAAG	TAGGACAT	ATGTCCTA	364	D10	GTTATCGA	GAAGTAGT	ACTACTTC
341	E07	TTCGACTC	AACACAAG	CTTGTGTT	365	E10	TGTCGTCG	GTTATCGA	TCGATAAC
342	F07	GTCGGTAA	TTCGACTC	GAGTCGAA	366	F10	CGTAACTG	TGTCGTCG	CGACGACA
343	G07	GTTCATTC	GTCGGTAA	TTACCGAC	367	G10	GCATGCCT	CGTAACTG	CAGTTACG
344	H07	AAGCAGTT	GTTCATTC	GAATGAAC	368	H10	TCGTACAC	GCATGCCT	AGGCATGC
345	A08	ATAAGCTG	AAGCAGTT	AACTGCTT	369	A11	CACAGGTG	TCGTACAC	GTGTACGA
346	B08	GCTTAGCG	ATAAGCTG	CAGCTTAT	370	B11	AGCAGTGA	CACAGGTG	CACCTGTG
347	C08	TTCCAACA	GCTTAGCG	CGCTAAGC	371	C11	ATTCCAGA	AGCAGTGA	TCACTGCT
348	D08	TACCGCAT	TTCCAACA	TGTTGGAA	372	D11	TCCTTGAG	ATTCCAGA	TCTGGAAT
349	E08	AGGCAATG	TACCGCAT	ATGCGGTA	373	E11	ATACCTAC	TCCTTGAG	CTCAAGGA
350	F08	GCCTCGTT	AGGCAATG	CATTGCCT	374	F11	AGACCATT	ATACCTAC	GTAGGTAT
351	G08	CACGGATC	GCCTCGTT	AACGAGGC	375	G11	CGTAAGCA	AGACCATT	AATGGTCT
352	H08	GAGACACG	CACGGATC	GATCCGTG	376	H11	TCTGTCAG	CGTAAGCA	TGCTTACG
353	A09	AGAGTAAG	GAGACACG	CGTGTCTC	377	A12	CACAGACT	TCTGTCAG	CTGACAGA
354	B09	AGTACGTT	AGAGTAAG	CTTACTCT	378	B12	GTCGCCTA	CACAGACT	AGTCTGTG
355	C09	AACGCTGC	AGTACGTT	AACGTACT	379	C12	TGCGCTCT	GTCGCCTA	TAGGCGAC
356	D09	GTAGAGCA	AACGCTGC	GCAGCGTT	380	D12	GCTATAAG	TGCGCTCT	AGAGCGCA
357	E09	TCCTGAGA	GTAGAGCA	TGCTCTAC	381	E12	CAACAACT	GCTATAAG	CTTATAGC
358	F09	CTGAATAG	TCCTGAGA	TCTCAGGA	382	F12	AGAGAATC	CTCTCACT	AGTGAGAG
359	G09	CAAGACTA	CTGAATAG	CTATTCAG	383	G12	TAATGGTC	AGACGAGC	GCTCGTCT
360	H09	GCACAGTA	CAAGACTA	TAGTCTTG	384	H12	GTTGTATC	TAATGGTC	GACCATTA

Index Primer Pair Strip Tube and Plate Maps

SureSelect XT HS2 Index Primer Pairs 1-16 (provided with 16 reaction kits) are supplied in a set of two 8-well strip tubes as detailed below.

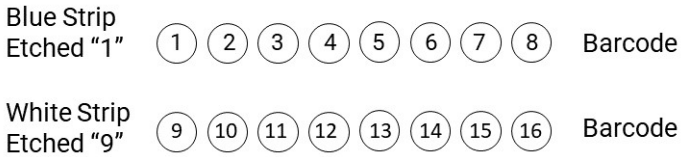


Figure 5 Map of the SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) strip tubes provided with 16 reaction kits

The blue strip contains Index Primer Pairs 1-8, with pair #1 supplied in the well proximal to the numeral ***1*** etched on the strip's plastic end tab.

The white strip contains Index Primer Pairs 9-16, with pair #9 supplied in the well proximal to the numeral ***9*** etched on the strip's plastic end tab.

When using the strip tube-supplied index primer pairs in the library preparation protocol, re-seal any unused wells using the fresh foil seal strips provided with the index strip tubes.

See Table 42 on page 68 through Table 45 on page 69 for plate maps showing positions of the SureSelect XT HS2 Index Primer Pairs provided with 96 reaction kits.

CAUTION The SureSelect XT HS2 Index Primer Pairs are provided in single-use aliquots. To avoid cross-contamination of libraries, use each well in only one library preparation reaction. Do not retain and re-use any residual volume for subsequent experiments.

Table 42 Plate map for SureSelect XT HS2 Index Primer Pairs 1-96, provided in orange plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Table 43 Plate map for SureSelect XT HS2 Index Primer Pairs 97-192, provided in blue plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	1110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Table 44 Plate map for SureSelect XT HS2 Index Primer Pairs 193-288, provided in green plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	193	201	209	217	225	233	241	249	257	265	273	281
B	194	202	210	218	226	234	242	250	258	266	274	282
C	195	203	211	219	227	235	243	251	259	267	275	283
D	196	204	212	220	228	236	244	252	260	268	276	284
E	197	205	213	221	229	237	245	253	261	269	277	285
F	198	206	214	222	230	238	246	254	262	270	278	286
G	199	207	215	223	231	239	247	255	263	271	279	287
H	200	208	216	224	232	240	248	256	264	272	280	288

Table 45 Plate map for SureSelect XT HS2 Index Primer Pairs 289-384, provided in red plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	289	297	305	313	321	329	337	345	353	361	369	377
B	290	298	306	314	322	330	338	346	354	362	370	378
C	291	299	307	315	323	331	339	347	355	363	371	379
D	292	300	308	316	324	332	340	348	356	364	372	380
E	293	301	309	317	325	333	341	349	357	365	373	381
F	294	302	310	318	326	334	342	350	358	366	374	382
G	295	303	311	319	327	335	343	351	359	367	375	383
H	296	304	312	320	328	336	344	352	360	368	376	384

Troubleshooting Guide

If yield of libraries is low

- ✔ The library preparation protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance of the highly viscous buffer and enzyme solutions used in the protocol. Be sure to adhere to all instructions when setting up the reactions.
- ✔ Ensure that the ligation master mix (see page 29) is kept at room temperature for 30–45 minutes before use.
- ✔ PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the PCR cycle number by 1 to 2 cycles.
- ✔ Performance of the solid-phase reversible immobilization (SPRI) purification step may be poor. Verify the expiration date for the vial of AMPure XP beads used for purification. Adhere to all bead storage and handling conditions recommended by the manufacturer. Ensure that the beads are kept at room temperature for at least 30 minutes before use. Use freshly-prepared 70% ethanol for each SPRI procedure.
- ✔ DNA elution during SPRI purification steps may be incomplete. Ensure that the AMPure XP beads are not overdried just prior to sample elution.
- ✔ Repeat library DNA concentration determination using a high-sensitivity assay. Visit the Automated Electrophoresis pages at agilent.com for information on the high-sensitivity DNA analysis kits available for your platform.

If solids observed in the End Repair-A Tailing Buffer

- ✔ Vortex the solution at high speed until the solids are dissolved. The observation of solids when first thawed does not impact performance, but it is important to mix the buffer until all solutes are dissolved.

If library fragment size is different than expected in electropherograms

- ✔ DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color and verify that you are using the correct bead volume at each purification step.

If low molecular weight adaptor-dimer peak is present in library electropherograms

✔ The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to the samples analyzed on page 42. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield. If excessive adaptor-dimers are observed, check the considerations below:

- Verify that the adaptor ligation protocol is being performed as directed on page 32. In particular, ensure that the Ligation master mix is mixed with the sample prior to adding the SureSelect XT HS2 RNA Adaptor Oligo Mix to the mixture. Do not add the Ligation master mix and the Adaptor Oligo Mix to the sample in a single step.
- Perform an additional round of purification using AMPure XP beads after the ligation step, as described on page 35 to page 35. Two serial purifications are used in the standard protocol for low-input or lower-quality libraries, but two serial purification steps may be implemented in the library preparation protocol for RNA input of any quantity and quality.

Quick Reference Protocol

An abbreviated summary of the protocol steps is provided below for experienced users. Use the complete protocol on page 17 to page 41 until you are familiar with all of the protocol details such as reagent mixing instructions and instrument settings.

Step	Summary of Conditions
	Poly-A mRNA Enrichment
Prepare and qualify RNA samples	Prepare 10–1000 ng total RNA in 25 µl nuclease-free water. Qualify integrity and adjust minimum RNA input as directed on page 17.
Denature and bind poly-A mRNA to oligo(dT) beads	25 µl total RNA sample + 25 µl Oligo(dT) Microparticles suspension Incubate in thermal cycler: 5 min @ 65°C, 1 min @ 4°C, Hold @ 4°C (RNA denaturation) Incubate 5 min at room temperature (bead binding)
Wash and elute bead-bound mRNA	Collect Oligo(dT) beads with magnetic stand, discard supernatant Wash beads with 200 µl Bead Washing Buffer Collect beads with magnetic stand, discard supernatant Resuspend beads with 25 µl Bead Elution Buffer Incubate in thermal cycler: 2 min @ 80°C, 1 min @ 4°C, Hold @ 4°C (RNA elution)
Re-bind poly-A mRNA to oligo(dT) beads	25 µl eluted RNA in Oligo(dT) bead suspension + 25 µl Bead Binding Buffer Incubate 5 min at room temperature (bead re-binding)
Wash and elute enriched mRNA	Collect Oligo(dT) beads with magnetic stand, discard supernatant Wash beads with 200 µl Bead Washing Buffer Collect beads with magnetic stand, discard supernatant Add 10 µl nuclease-free H_2O, retaining beads and liquid in sample well Keep on ice
	RNA Fragmentation and cDNA Preparation
Fragment mRNA and prime cDNA synthesis	10 µl bead-bound enriched poly-A mRNA+ 10 µl 2× Priming Buffer Incubate in thermal cycler: 4 min @ 94°C, 1min @ 4°C, Hold @ 4°C Collect Oligo(dT) beads with magnetic stand, **transfer 20 µl supernatant to fresh well**
Synthesize first-strand cDNA	20 µl primed mRNA fragments + 8.5 µl First Strand Master Mix Incubate in thermal cycler: 10 min @ 25°C, 40 min @ 37°C, Hold @ 4°C

Step	Summary of Conditions
Synthesize second-strand cDNA	28.5 µl first-strand cDNA+ 25 µl Second Strand Enzyme Mix + 5 µl Second Strand Oligo Mix Incubate in thermal cycler: 60 min @ 16°C, Hold @ 4°C
Purify cDNA	58.5 µl cDNA sample + 105 µl AMPure XP bead suspension Elute cDNA in 52 µl nuclease-free H_2O, removing 50 µl to fresh well Keep on ice
	Library Prep
Prepare Ligation master mix	Per reaction: 23 µl Ligation Buffer + 2 µl T4 DNA Ligase Keep at room temperature 30–45 min before use
Prepare End-Repair/dA-Tailing master mix	Per reaction: 16 µl End Repair-A Tailing Buffer + 4 µl End Repair-A Tailing Enzyme Mix Keep on ice
End-Repair and dA-Tail the DNA fragments	50 µl cDNA fragments + 20 µl End Repair/dA-Tailing master mix Incubate in thermal cycler: 15 min @ 20°C, 15 min @ 72°C, Hold @ 4°C
Ligate adaptor	70 µl DNA sample + 25 µl Ligation master mix +5 µl SureSelect XT HS2 RNA Adaptor Oligo Mix Incubate in thermal cycler: 30 min @ 20°C, Hold @ 4°C
Purify DNA	100 µl DNA sample + 80 µl AMPure XP bead suspension Elute DNA in 35 µl nuclease-free H_2O, removing 34 µl to fresh well (For libraries from input RNA <100 ng RNA or RIN 6-8, do two serial purifications as directed on page 34) Keep on ice
Prepare PCR master mix	Per reaction: 10 µl 5× Herculase II Reaction Buffer with dNTPs + 1 µl Herculase II Fusion DNA Polymerase Keep on ice
Amplify the purified DNA	34 µl purified DNA + 11 µl PCR master mix + 5 µl assigned SureSelect XT HS2 Index Primer Pair Amplify in thermal cycler using program on page 37
Purify amplified DNA	50 µl amplified DNA + 50 µl AMPure XP bead suspension Elute DNA in 15 µl 1× Low TE Buffer.
Quantify and qualify DNA	Analyze quantity and quality using TapeStation, Bioanalyzer, or Fragment Analyzer System

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In This Book

This guide contains instructions for using the SureSelect XT HS2 mRNA Reagent Kits to prepare NGS libraries for the Illumina platform.

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