SureSelect XT HS2 RNA System with Pre-Capture Pooling

Strand-Specific RNA Library Preparation and Target Enrichment for the Illumina Platform

Protocol

Version B0, August 2021

SureSelect platform manufactured with Agilent SurePrint Technology

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Notices

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In this Guide...

This guide provides an optimized protocol for preparation of pre-captured pooled NGS sequencing libraries, prepared using the SureSelect XT HS2 RNA system.

1 Before You Begin

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

2 Preparation of Input RNA and Conversion to cDNA

This chapter describes the steps to prepare, qualify and fragment the RNA samples, then convert RNA to cDNA fragments.

3 Library Preparation

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

4 Hybridization and Capture

This chapter describes the steps to hybridize and capture the prepared cDNA library using a SureSelect or ClearSeq probe capture library.

5 Post-Capture Sample Processing for Multiplexed Sequencing

This chapter describes the steps for post-capture amplification and guidelines for sequencing sample preparation.

6 Reference

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

What's New in Version B0

- Support for SureSelect XT HS PreCap Human All Exon V8 Probe. See Table 2 on page 13 for ordering information. See Table 25 on page 48 for pre-capture pooling recommendations. See Table 27 on page 50 for the hybridization thermal cycler program recommended for this probe with the SureSelect XT HS2 RNA system. Also see troubleshooting information on page 94 and updates to the *Quick Reference Protocol* on page 96.
- Updates to description of pre-capture pooling probe configurations (see Table 3 on page 14) and of flat strip caps in Table 7 on page 17.
- Minor updates to "Preparation of Input RNA and Conversion to cDNA" instructions including addition of reagent vial cap colors and new *Note* on page 27.
- Updates to "Hybridization and Capture" instructions. Updates include provision of two separate hybridization thermal cycler programs (Table 27 and Table 28 on page 50), addition of hybridization temperature considerations for probes designed for use with the SureSelect XT system (Table 28 on page 50) and additional minor changes.
- Updates to "Post-Capture Sample Processing for Multiplexed Sequencing" instructions. Updates include changes to Table 40 on page 69, revised guidance for RNA sequence processing using AGeNT on page 75, and additional minor changes.

What's New in Version A1

- Updates to index pair sequence tables (page 82 through page 89) including updates to P5 index platform descriptions and correction of well position typographical errors
- Updates to downstream sequencing support information (see Table 40 on page 69 and *Note* on page 81)
- Updates to thawing conditions in Table 14 on page 32

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 RNA workflow for the preparation of NGS-ready libraries is summarized in Figure 1.

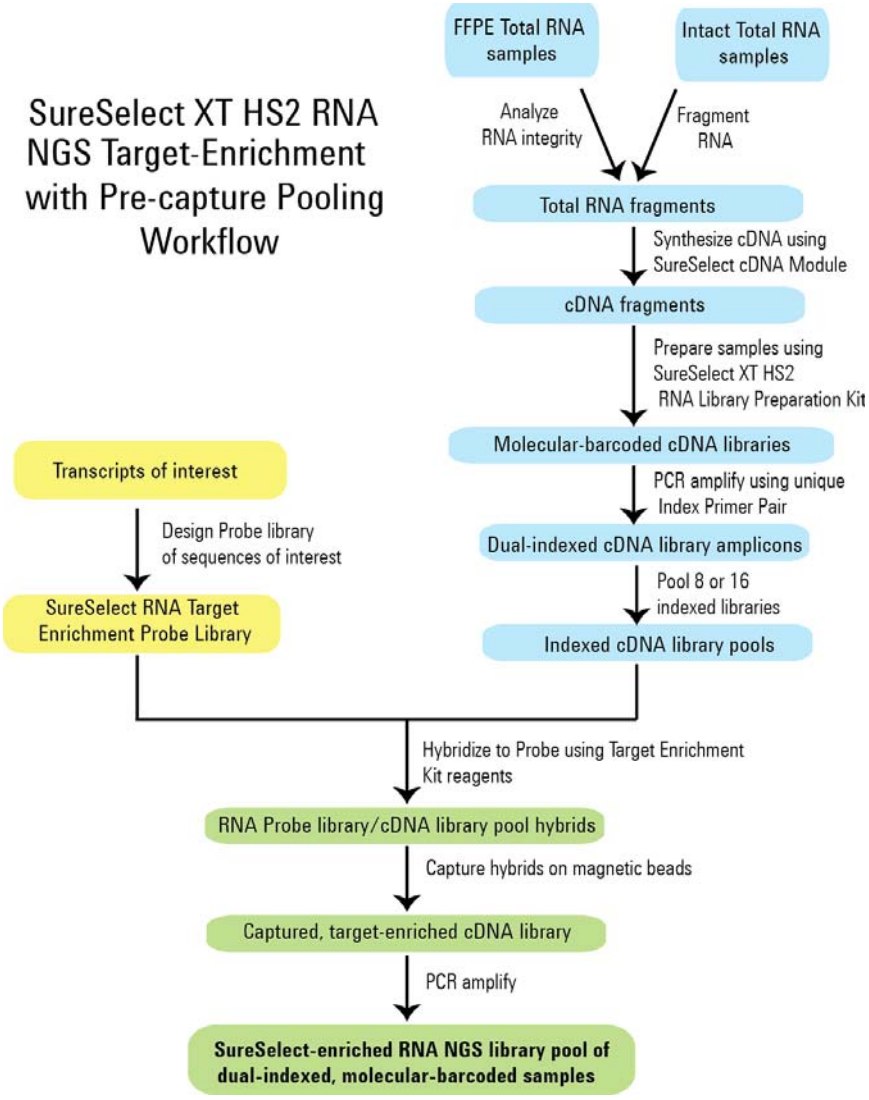


Figure 1 Overall target-enriched RNA sequencing sample preparation workflow.

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

To determine the materials required for your unique needs, first see Table 1 below for the required Library Preparation Kit and Target Enrichment Kit ordering information. Second, select a pre-capture pooling-formulated target enrichment probe from Table 2. Third, refer to Table 4 through Table 7 for additional materials needed to complete the protocols.

Table 1 Pre-Capture Pooling-Compatible SureSelect XT HS2 RNA Reagent Kits

Description	Kit Part Number
SureSelect XT HS2 RNA Library Preparation Kit for ILM, 96 Reactions* (select one)	G9993A (with Index Pairs 1–96) G9993B (with Index Pairs 97–192) G9993C (with Index Pairs 193–288) G9993D (with Index Pairs 289–384)
AND	
SureSelect XT HS2 RNA Target Enrichment Kit, 12 Hybs†	G9994A

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

† The 12-Hyb Target Enrichment Kit supports target enrichment for 96 samples pre-capture pooled using the configuration of either 8 samples/pool or 16 samples/pool as recommended for each compatible probe.

Table 2 Compatible Probes

Probe Capture Library	Design Target	Ordering Information
Custom Probes*		
SSEL PreCap Custom Tier1 1–499 kb (6 Hybs or 30 Hybs)†		Please contact the SureSelect support team (see page 2) or your local representative for assistance with custom probe design and ordering for RNA library target enrichment.
SSEL PreCap Custom Tier2 0.5 –2.9 Mb (6 Hybs or 30 Hybs)†		
SSEL PreCap Custom Tier3 3 –5.9 Mb (6 Hybs or 30 Hybs)†		
SSEL PreCap Custom Tier4 6 –11.9 Mb (6 Hybs or 30 Hybs)†		
SSEL PreCap Custom Tier5 12–24 Mb (6 Hybs or 30 Hybs)†		
Pre-designed Probes		
SureSelect XT HS PreCap Human All Exon V8 (12 Hybs)†	Genome	Agilent p/n 5191-6877
SureSelect XT HS PreCap Human All Exon V7 (12 Hybs)†	Genome	Agilent p/n 5191-5735
SureSelect XT2 Clinical Research Exome V2 (12 Hybs)†	Genome	Agilent p/n 5190-9501
SureSelect XT2 Mouse All Exon (12 Hybs)†	Genome	Agilent p/n 5190-4682
ClearSeq Inherited Disease XT2 (12 Hybs)†	Genome	Agilent p/n 5190-7525
ClearSeq Comprehensive Cancer XT2 (6 Hybs)†	Genome	Agilent p/n 5190-8018
Pre-designed Probes customized with additional *Plus* custom content		
SureSelect XT2 Clinical Research Exome V2 Plus 1 (12 Hybs)†	Genome	Please visit the SureDesign website to design the customized *Plus* content and obtain ordering information. Contact the SureSelect support team (see page 2) or your local representative if you need assistance.
SureSelect XT2 Clinical Research Exome V2 Plus 2 (12 Hybs)†	Genome	
ClearSeq Comprehensive Cancer Plus XT2 (6 Hybs)†	Genome	
ClearSeq Inherited Disease Plus XT2 (12 Hybs)†	Genome	

* Custom Probes designed August 2020 or later are produced using an updated manufacturing process; design-size Tier is shown on labeling for these products. Custom Probes designed and ordered prior to August 2020 may be reordered, with these probes produced using the legacy manufacturing process; design-size Tier is not shown on labeling for the legacy-process products. Custom Probes of both types use the same optimized target enrichment protocols detailed in this publication.

† See Table 3 on page 14 for samples processed and supported run configuration details for each probe pack size. For example, the 6-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 16 samples/pool (6 hybs × 16 samples/hyb). Probe vials contain enough reagents for the run configuration listed in Table 3 using the run setup on page 52.

Table 3 Supported Run Configurations

Probe Type	Pack Size	Samples Pooled per Hybridization	Total Samples Processed	Minimum Supported Run Size
PreCap Custom Probes	6 Hybs	16	96	1 run x 6 hybs/run
	30 Hybs	16	480	5 runs x 6 hybs/run
ClearSeq Comprehensive Cancer XT2	6 Hybs	16	96	1 run x 6 hybs/run
SureSelect XT HS PreCap Human All Exon V8 SureSelect XT HS PreCap Human All Exon V7 SureSelect XT2 Clinical Research Exome V2 SureSelect XT2 Mouse All Exon ClearSeq Inherited Disease XT2	12 Hybs	8	96	2 runs x 6 hybs/run

Table 4 Required Reagents

Description	Vendor and Part Number
1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930
AMPure XP Kit 5 ml 60 ml 450 ml	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
Dynabeads MyOne Streptavidin T1 2 ml 10 ml 50 ml	Thermo Fisher Scientific p/n 65601 p/n 65602 p/n 65604D
QPCR Human Reference Total RNA (optional control input RNA)	Agilent p/n 750500

CAUTION Sample volumes exceed 0.2 ml in certain steps of this protocol. Make sure that the plasticware used with the selected thermal cycler holds ≥0.25 ml per well.

Table 5 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, domed	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
96-well plate mixer	Eppendorf ThermoMixer C, p/n 5382000023 and Eppendorf SmartBlock 96 PCR, p/n 5306000006, or equivalent
Small-volume spectrophotometer	NanoDrop 2000, Thermo Fisher Scientific p/n ND-2000 or equivalent
Vacuum concentrator	Savant SpeedVac, model DNA120, 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 6 Nucleic Acid Analysis Platform Options--Select One

Description	Vendor and Part Number
Agilent 4200/4150 TapeStation	Agilent p/n G2991AA/G2992AA
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
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585
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
High Sensitivity DNA Kit	Agilent p/n 5067-4626
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
HS NGS Fragment Kit (1-6000 bp)	p/n DNF-474-0500

Optional Materials

Table 7 Supplier Information for Optional Materials

Description	Vendor and Part Number	Purpose
Tween 20	Sigma-Aldrich p/n P9416-50ML	Sequencing library storage (see page 67)
8× flat strip caps	Consult the thermal cycler manufacturer's recommendations	Sealing wells for protocol steps performed outside of the thermal cycler*
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

* Flat strip caps may be used instead of domed strip caps for protocol steps performed outside of the thermal cycler. Adhesive film may be applied over the flat strip caps for improved sealing properties.

2 Preparation of Input RNA and Conversion to cDNA

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

The protocol is compatible with both intact RNA prepared from fresh or fresh frozen samples and lower-quality RNA prepared from FFPE samples. For FFPE-derived RNA samples, begin the protocol using "Step 1A. Prepare and qualify FFPE RNA samples" on page 21. For intact RNA samples, begin the protocol using "Step 1B. Prepare and fragment intact RNA samples" on page 24.

RNA sequencing library preparation requires RNA fragments sized appropriately for the NGS workflow. In this section of the protocol, intact total RNA samples are chemically-fragmented by treatment with metal ions present in the 2X Priming Buffer at elevated temperature. FFPE-derived RNA samples are already sufficiently fragmented. The FFPE samples must be combined with the same 2X Priming Buffer, but the mixtures are held on ice, preventing further fragmentation of the FFPE-derived RNA.

Protocols in this section for both intact RNA and FFPE sample types are applicable to either 2 x 100 bp or 2 x 150 bp read-length sequencing.

The protocol steps in this section use the components listed in Table 8. Thaw and mix each component as directed in Table 8 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 28. *Do not freeze the beads at any time.*

Table 8 Reagents thawed before use in protocol

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 23 (FFPE RNA) **OR** page 25 (intact RNA)
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 26
Second Strand Enzyme Mix (bottle)	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	page 27
Second Strand Oligo Mix (tube with yellow cap)	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	page 27

* 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 1A. Prepare and qualify FFPE RNA samples

The instructions in this section are for FFPE-derived RNA samples. For intact (non-FFPE) RNA samples, instead follow the instructions in "Step 1B. Prepare and fragment intact RNA samples" on page 24.

Prepare total RNA from each FFPE sample in the run. The library preparation protocol requires 10–200 ng of FFPE total RNA in a 10 µl volume of nuclease-free water.

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.

Before you begin the library preparation protocol, assess the initial quality of each sample in order to determine the appropriate reaction conditions at several steps in the workflow. Use the steps below to qualify each FFPE total RNA sample.

1 Use a small-volume spectrophotometer to determine sample absorbance at 260 nm, 280 nm, and 230 nm. Determine the RNA concentration and the 260/280 and 260/230 absorbance ratio values for the sample.

  High-quality RNA samples are indicated by values of approximately 1.8 to 2.0 for both ratios. Ratios with significant deviation from 2.0 indicate the presence of organic or inorganic contaminants, which may require further purification or may indicate that the sample is not suitable for use in RNA target enrichment applications.

2 Examine the starting size distribution of RNA in the sample using one of the RNA qualification systems described in Table 9. Select the specific assay appropriate for your sample based on the RNA concentration determined in step 1 on page 21.

Determine the DV200 (percentage of RNA in the sample that is >200 nt) using the analysis mode described in Table 9. RNA molecules must be >200 nt for efficient conversion to cDNA library.

Table 9 RNA qualification platforms

Analysis Instrument	RNA Qualification Assay	Analysis to Perform
4200/4150 TapeStation	RNA ScreenTape or High Sensitivity RNA ScreenTape	Region analysis using TapeStation Analysis Software
2100 Bioanalyzer	RNA 6000 Pico Chip or NanoChip	Smear/Region analysis using 2100 Expert Software
5200 Fragment Analyzer	RNA Kit (15NT) or HS RNA Kit (15NT)	Analysis using ProSize Data Analysis Software

NOTE Grading of FFPE RNA quality by RNA Integrity Number (RIN) is not recommended for this application.

3 Grade each RNA sample based on the percentage of RNA in the sample >200 nucleotides, according to Table 10.

Table 10 Classification of FFPE RNA samples based on starting RNA size

Grade	DV200	Recommended input amount	Minimum input amount
Good FFPE RNA	>50%	200 ng	10 ng
Poor FFPE RNA	20% to 50%	200 ng	50 ng*
Inapplicable FFPE RNA	<20%	Not recommended for further processing	

* For optimal results, prepare libraries from poor-grade FFPE RNA samples using a minimum of 50 ng input RNA. Libraries may be prepared from 10–50 ng poor-grade FFPE RNA with potential negative impacts on yield or NGS performance.

4 Place 10 µl of each sample, containing 10–200 ng of FFPE total RNA in nuclease-free water, into wells of a thermal cycler-compatible strip tube or PCR plate.

Poor-quality FFPE samples should contain at least 50 ng RNA.

5 Add 10 µl of 2X Priming Buffer (purple cap) to each sample well.

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 then place the RNA samples on ice.

NOTE

All samples, including highly degraded FFPE samples, must be combined with 2X Priming Buffer, which supplies the random primers for cDNA synthesis. FFPE RNA samples are not subjected to the high-temperature incubation step used for fragmentation in this buffer.

7 Proceed immediately to "Step 2. Synthesize first-strand cDNA" on page 26.

Step 1B. Prepare and fragment intact RNA samples

The instructions in this section are for intact RNA prepared from fresh or fresh frozen samples. For FFPE-derived RNA samples, instead follow the instructions in "Step 1A. Prepare and qualify FFPE RNA samples" on page 21.

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.

The 2X Priming Buffer used 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 Prepare total RNA from each sample in the run. The library preparation protocol requires 10–200 ng of intact total RNA in a 10 µl volume of nuclease-free water.

   Verify the RNA concentration and quality using a small volume spectrophotometer and one of the RNA qualification platforms listed in Table 6 on page 16.

2 Preprogram a thermal cycler with the program in Table 11. Immediately pause the program, and keep paused until samples are loaded in step 6.

Table 11 Thermal cycler program for fragmentation of intact RNA 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.

NOTE

When using the SureCycler 8800 thermal cycler, the heated lid may be left on (default setting) throughout the RNA library preparation incubation steps. The heated lid must be on during the amplification and hybridization steps on page 40, page 50 and page 59.

3 Place 10 µl of each sample, containing 10–200 ng total RNA in nuclease-free water, into wells of a thermal cycler-compatible strip tube or PCR plate.

4 Add 10 µl of 2X Priming Buffer (purple cap) to each sample well.

5 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.

6 Place the samples in the thermal cycler, and resume the thermal cycling program in Table 11 for RNA fragmentation.

7 Once the thermal cycler program in Table 11 reaches the 4°C Hold step, transfer the fragmented RNA sample plate or strip tube from the thermal cycler to ice or a cold block. Proceed immediately to "Step 2. Synthesize first-strand cDNA" on page 26.

Step 2. 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 12. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 12 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 (amber tube/cap) 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 12.

Step 3. 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 12 begins the 4°C hold step, transfer the samples to ice.

2 Preprogram the thermal cycler with the program in Table 13. Immediately pause the program, and keep paused until samples are loaded in step 7.

Table 13 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 (bottle) and of Second Strand Oligo Mix (yellow cap) 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 13.

NOTE The AMPure XP beads used in the next step must be equilibrated to room temperature for at least 30 minutes before use.

Step 4. 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. The complete RNA Library Preparation protocol requires 1.2 mL of fresh 70% ethanol per sample and the Target Enrichment protocol requires an additional 0.4 mL of fresh 70% ethanol per sample.

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 Transfer 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 molecular-barcoded library is prepared.

Protocol steps in this section use the components listed in Table 14. Thaw and mix each component as directed in Table 14 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 37. *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 24 samples (including excess) are shown in tables as examples.

Table 14 Reagents thawed before use in protocol

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
Ligation Buffer (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 33
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 33
End Repair-A Tailing Buffer (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 35
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 35
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 36

Step 1. Prepare the Ligation master mix

Prepare the Ligation master mix to allow equilibration to room temperature before use on page 36. 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 15.

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 36.

Table 15 Preparation of Ligation master mix

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

* 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 with the program in Table 16. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 16 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 17.

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 17 Preparation of End Repair/dA-Tailing master mix

Reagent	Volume for 1 reaction	Volume for 24 reactions (includes excess)
End Repair-A Tailing Buffer (bottle)	16 µl	400 µl
End Repair-A Tailing Enzyme Mix (orange cap)	4 µl	100 µl
Total	**20 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 16.

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 with the program in Table 18. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 18 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 DNA sample (approximately 70 µl), add 25 µl of the Ligation master mix that was prepared on page 33 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 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 XT HS2 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 18.

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

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 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 35 µl nuclease-free water 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 approximately 5 minutes, until the solution is clear.

17 Transfer the cleared supernatant (approximately 34 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 34-µl supernatant volume at this step; transfer the maximum possible amount of supernatant for further processing. To maximize recovery, transfer the cleared supernatant to a fresh well in two rounds of pipetting, using a P20 pipette set at 17 µl.

Step 5. Amplify the adaptor-ligated cDNA library

This step uses the components listed in Table 19. Before you begin, thaw the reagents listed below and keep on ice.

Table 19 Reagents for pre-capture 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 41
5× Herculase II Buffer with dNTPs (clear cap)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	page 41
SureSelect XT HS2 Index Primer Pairs	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR),* –20°C	Vortexing	page 41

* Unique indexing primer pairs are provided in individual wells of 96-well plates.

1 Determine the appropriate index pair assignment for each sample. See Table 48 on page 82 through Table 55 on page 89 for nucleotide 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, 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 20. Immediately pause the program, and keep paused until samples are loaded in step 6.

Table 20 Pre-Capture PCR Thermal Cycler Program*

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10–14 (See Table 21 for RNA input-based cycle number recommendations)	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 21 Pre-capture PCR cycle number recommendations

Quality of Input RNA	Quantity of Input RNA	Cycle Number
Intact RNA	100 to 200 ng	10 cycles
	50 ng	11 cycles
	10 ng	12 cycles
Good quality FFPE RNA (DV200 >50%)	100 to 200 ng	12 cycles
	50 ng	13 cycles
	10 ng	14 cycles
Poor quality FFPE RNA (DV200 20% to 50%)	100 to 200 ng	13 cycles
	50 ng	14 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 pre-capture PCR reaction mix, as described in Table 22, on ice. Mix well on a vortex mixer.

Table 22 Preparation of Pre-Capture PCR Reaction Mix

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

4 Add 11 µl of the PCR reaction mixture prepared in Table 22 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 20 to bring the temperature of the thermal block to 98°C. Once the cycler has reached 98°C, 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 nuclease-free water 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 Transfer 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.

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.

Step 7. Assess quality and quantity

Analyze each sample using one of the platforms listed in Table 23. Follow the instructions in the linked user guide provided for each assay in Table 23, after reviewing the SureSelect library qualification steps on page 45. 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 24 for fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided to illustrate typical results for libraries prepared from either high-quality or FFPE RNA samples.

Table 23 Pre-capture 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 24 Pre-capture library qualification guidelines

Input RNA type	Expected library DNA fragment size peak position	NGS read lengths supported
High-quality RNA or FFPE 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 example electropherograms in this section. See *Troubleshooting* on page 93 for additional considerations.

1 Set up the instrument as instructed in the appropriate user guide (links provided in Table 23).

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 24 for guidelines). Sample TapeStation system electropherograms are shown for libraries prepared from high-quality RNA in Figure 2 and from FFPE RNA in Figure 3.

   Electropherograms obtained using the other analysis platform options listed in Table 23 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.

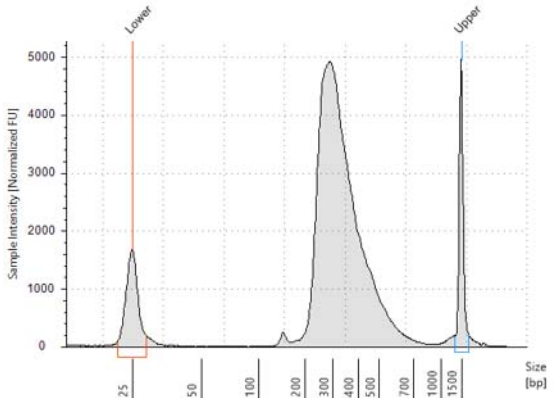


Figure 2 Pre-capture library prepared from high-quality RNA sample (Human Reference Total RNA) analyzed using a D1000 ScreenTape.

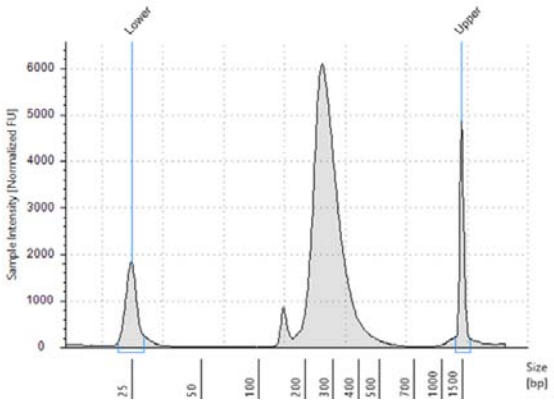


Figure 3 Pre-capture library prepared from a typical FFPE RNA sample analyzed using a D1000 ScreenTape.

4 Hybridization and Capture

This chapter describes the steps to pool the indexed cDNA libraries and then hybridize the pooled cDNA libraries with a target-specific probe. Pools of 8 or 16 indexed samples are hybridized to the appropriate probe and the targeted molecules are captured on streptavidin-coated beads.

The recommended number of indexes that may be combined for hybridization varies for different probes. See Table 25 for pooling recommendations.

The standard single-day protocol includes the hybridization step immediately followed by capture and amplification steps. If required, the hybridized samples may be held overnight with capture and amplification steps completed the following day by using the simple protocol modifications noted on page 51.

CAUTION The ratio of probe to cDNA library is critical for successful capture.

Step 1. Pool indexed cDNA libraries for hybridization

In this step, you pool the indexed cDNA library samples, then adjust the pool volume to 12 µl, before hybridization to the probe.

Each hybridization reaction requires a total of either 1.2 µg or 600 ng indexed cDNA, made up of equal amounts of 8 or 16 individual libraries, based on the specific probe design used for hybridization. See Table 25 for probe-specific cDNA library pool composition recommendations.

Table 25 Pre-capture pooling recommendations

Probe description	Total amount of indexed gDNA pool used for hybridization	Number of indexed gDNA libraries per pool	Amount of each gDNA library in pool
SureSelect XT HS PreCap Human All Exon V8	1.2 µg	8	150 ng
SureSelect XT HS PreCap Human All Exon V7	600 ng	8	75 ng
SureSelect XT2 Mouse All-Exon	600 ng	8	75 ng
SureSelect XT2 Clinical Research Exome V2	600 ng	8	75 ng
ClearSeq Inherited Disease XT2	600 ng	8	75 ng
ClearSeq Comprehensive Cancer XT2	600 ng	16	37.5 ng
SSEL PreCap Custom Probes	600 ng	16	37.5 ng

1 For each capture reaction pool, combine the appropriate volume of each indexed cDNA library sample in one well of a strip tube or PCR plate.

   Each final capture reaction pool should contain either 1.2 µg or 600 ng indexed cDNA (see Table 25).

2 Use a vacuum concentrator, held at ≤ 45°C, to reduce the volume in each well to <12 µl.

   Avoid completely drying the sample. Over-drying the indexed library pool negatively impacts target enrichment.

3 Add sufficient nuclease-free water to each concentrated cDNA pool to bring the final well volume to 12 µl.

4 Cap the wells, then vortex the strip tube or plate vigorously for 30 seconds. Spin in a centrifuge or mini-plate spinner to collect the liquid, then keep the samples on ice until use on page 51.

Step 2. Hybridize cDNA libraries to the probe

In this step, the indexed cDNA library pools are hybridized to a target-specific probe. This step uses the components listed in Table 26. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 26 Reagents for Hybridization

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect XT HS2 Blocker Mix (blue cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	page 51
SureSelect RNase Block (purple cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	page 51
SureSelect Fast Hybridization Buffer (bottle)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw and keep at Room Temperature	page 52
Probe	–80°C	Thaw on ice	page 52

1 Preprogram a thermal cycler (with heated lid ON) with the program in Table 27 for the SureSelect XT HS Human All Exon V8 Probe or in Table 28 for all other probes. Immediately pause the program, and keep paused until samples are loaded in step 3 on page 51.

Table 27 Hybridization program **for SureSelect XT HS Human All Exon V8 Probe** *

Segment #	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	65°C	10 minutes
3	1	65°C	1 minute (Pause cycler here for reagent addition; see step 6 on page 53)
4	60	65°C	1 minute
		37°C	3 seconds
5	1	65°C	60 minutes
6	1	65°C	Hold briefly until ready to begin capture steps on page 55

* Use a reaction volume setting of 30 µl (final volume of hybridization reactions in Segment 4).

Table 28 Hybridization program **for all other probes** *

Segment #	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	65°C	10 minutes
3	1	65°C	1 minute (Pause cycler here for reagent addition; see step 6 on page 53)
4	60	65°C†	1 minute
		37°C	3 seconds
5	1	65°C†	Hold briefly until ready to begin capture steps on page 55

* Use a reaction volume setting of 30 µl (final volume of hybridization reactions during cycling in Segment 4).

† Hybridization at 65°C is optimal for probes designed for the SureSelect XT HS2/XT HS/XT Low Input platforms. Reducing the hybridization temperature (Segments 4 and 5) may improve performance for probes designed for the SureSelect XT2 platform, including SureSelect XT2 Human All Exon V6 (62.5°C), SureSelect XT2 Clinical Research Exome V2 (62.5°C) and custom probes originally designed for use with SureSelect XT2 system (60°C–65°C).

NOTE

The Hybridization reaction may be run overnight with the following protocol modifications:

- In the final segment of the thermal cycler program (Table 27 or Table 28), replace the 65°C Hold step with a 21°C Hold step.
- The hybridized samples may be held at 21°C for up to 16 hours. Begin the capture preparation steps on page 54 on day 2, after the overnight hold.

2 To each well containing 12 µl of pooled DNA libraries add 5 µl of SureSelect XT HS2 Blocker Mix (blue cap). Cap the wells then vortex at high speed for 5 seconds. Spin briefly to collect the liquid and release any bubbles.

CAUTION

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

3 Transfer the sealed sample plate or strip to the thermal cycler and resume the thermal cycling program (Table 27 or Table 28 on page 50) allowing the cycler to complete Segments 1 and 2 of the program.

Important: The thermal cycler must be paused during Segment 3 to allow additional reagents to be added to the hybridization wells in step 6 on page 53.

During Segments 1 and 2 of the thermal cycling program, begin preparing the additional reagents as described in step 4 and step 5 below. If needed, you can finish these preparation steps after pausing the thermal cycler in Segment 3.

4 Prepare a 25% solution of SureSelect RNase Block (1 part RNase Block to 3 parts water), according to Table 29. Prepare the amount required for the number of hybridization reactions in the run, plus excess. Mix well and keep on ice.

Table 29 Preparation of RNase Block solution

Reagent	Volume for 1 Hyb reaction	Volume for 6 Hyb reactions (includes excess)
SureSelect RNase Block	0.5 µl	3.5 µl
Nuclease-free water	1.5 µl	10.5 µl
Total	**2 µl**	**14 µl**

NOTE

Prepare the mixture described in step 5, below, just before pausing the thermal cycler in Segment 3. Keep the mixture at room temperature briefly until the mixture is added to the DNA samples in step 6 on page 53. Do not keep solutions containing the probe at room temperature for extended periods.

5 Prepare the Probe Hybridization Mix appropriate for your probe design size. Use Table 30 for probes ≥3 Mb or Table 31 for probes <3 Mb. For custom probes, see the probe tube label for design size range.

Combine the listed reagents at room temperature. Mix well by vortexing at high speed for 5 seconds then spin down briefly. Proceed immediately to step 6.

Table 30 Preparation of Probe Hybridization Mix for **probes≥3 Mb**

Reagent	Volume for 1 Hyb reaction	Volume for 6 Hyb reactions (includes excess)
25% RNase Block solution (from step 4)	2 µl	14 µl
Probe (with design ≥3 Mb)	5 µl	35 µl
SureSelect Fast Hybridization Buffer	6 µl	42 µl
Total	**13 µl**	**91 µl**

Table 31 Preparation of Probe Hybridization Mix for **probes<3 Mb**

Reagent	Volume for 1 Hyb reaction	Volume for 6 Hyb reactions (includes excess)
25% RNase Block solution (from step 4)	2 µl	14 µl
Probe (with design<3 Mb)	2 µl	14 µl
SureSelect Fast Hybridization Buffer	6 µl	42 µl
Nuclease-free water	3 µl	21 µl
Total	**13 µl**	**91 µl**

6 Once the thermal cycler starts Segment 3 (1 minute at 65°C), pause the program. With the cycler paused, and while keeping the DNA + Blocker samples in the cycler, transfer 13 µl of the room-temperature Probe Hybridization Mix from step 5 to each sample well.

Mix well by pipetting up and down slowly 8 to 10 times.

The hybridization reaction wells now contain approximately 30 µl.

7 Seal the wells with fresh domed strip caps. Make sure that all wells are completely sealed. Vortex briefly, then spin the plate or strip tube briefly to remove any bubbles from the bottom of the wells. Immediately return the plate or strip tube to the thermal cycler.

8 Resume the thermal cycling program to allow hybridization of the prepared DNA samples to the probe.

CAUTION Wells must be adequately sealed to minimize evaporation, or your results can be negatively impacted.

Before you do the first experiment, make sure the plasticware and capping method are appropriate for the thermal cycler. Check that no more than 4 µl is lost to evaporation under the conditions used for hybridization.

Step 3. Prepare streptavidin-coated magnetic beads

The remaining hybridization capture steps use the reagents in Table 32.

NOTE

If performing same-day hybridization and capture, begin the bead preparation steps below approximately one hour after starting hybridization in step 8 on page 53. If performing next-day capture after an overnight hold at 21°C, begin the bead preparation steps below on day 2, just before you are ready to start the capture steps on page 55.

Table 32 Reagents for Capture

Kit Component	Storage Location	Where Used
SureSelect Binding Buffer	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), RT	page 54
SureSelect Wash Buffer 1	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), RT	page 55
SureSelect Wash Buffer 2	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), RT	page 55
Dynabeads MyOne Streptavidin T1 Beads	4°C	page 54

1. Vigorously resuspend the vial of streptavidin beads on a vortex mixer. The magnetic beads settle during storage.
2. For each hybridization sample, add 50 µl of the resuspended beads to wells of a fresh PCR plate or a strip tube.
3. Wash the beads:
   a. Add 200 µl of SureSelect Binding Buffer.
   b. Mix by pipetting up and down 20 times or cap the wells and vortex at high speed for 5–10 seconds then spin down briefly.
   c. Put the plate or strip tube into a magnetic separator device.
   d. Wait 5 minutes or until the solution is clear, then remove and discard the supernatant.
   e. Repeat step a through step d two more times for a total of 3 washes.
4. Resuspend the beads in 200 µl of SureSelect Binding Buffer.

NOTE

If you are equipped for higher-volume magnetic bead captures, the streptavidin beads may instead be batch-washed in a microcentrifuge tube or conical vial.

Step 4. Capture the hybridized DNA using streptavidin-coated beads

1 After all streptavidin bead preparation steps are complete, and with the hybridization thermal cycling program in the final hold segment (see Table 27 or Table 28 on page 50), transfer the samples to room temperature.

2 Immediately transfer the entire volume (approximately 30 µl) of each hybridization mixture to wells containing 200 µl of washed streptavidin beads using a multichannel pipette.

   Pipette up and down 5–8 times to mix then seal the wells with fresh domed caps.

3 Incubate the capture plate or strip tube on a 96-well plate mixer, mixing vigorously (at 1400–1900 rpm), for 30 minutes at room temperature.

   Make sure the samples are properly mixing in the wells.

4 During the 30-minute incubation for capture, prewarm SureSelect Wash Buffer 2 at 70°C as described below.

   a Place 200-µl aliquots of Wash Buffer 2 in wells of a fresh 96-well plate or strip tubes. Aliquot 6 wells of buffer for each hybridization reaction in the run.

   b Cap the wells and then incubate in the thermal cycler, held at 70°C, until used in step 9.

5 When the 30-minute capture incubation period initiated in step 3 is complete, spin the samples briefly to collect the liquid.

6 Put the plate or strip tube in a magnetic separator to collect the beads. Wait until the solution is clear, then remove and discard all of the supernatant.

7 Resuspend the beads in 200 µl of SureSelect Wash Buffer 1. Mix by pipetting up and down 15–20 times, until beads are fully resuspended.

8 Put the plate or strip tube in the magnetic separator. Wait for the solution to clear (approximately 1 minute), then remove and discard all of the supernatant.

CAUTION It is important to maintain bead suspensions at 70°C during the washing procedure below to ensure specificity of capture.

Make sure that the SureSelect Wash Buffer 2 is pre-warmed to 70°C before use.

Do not use a tissue incubator, small benchtop incubator, or other devices with significant temperature fluctuations, for the incubation steps.

9 Remove the plate or strip tubes from the magnetic separator and transfer to a rack at room temperature. Wash the beads with Wash Buffer 2, using the protocol steps below.

a Resuspend the beads in 200 µl of 70°C prewarmed Wash Buffer 2. Pipette up and down 15–20 times, until beads are fully resuspended.

b Carefully seal the wells with fresh caps and then vortex at high speed for 8 seconds. Spin the plate or strip tube briefly to collect the liquid without pelleting the beads.

Make sure the beads are in suspension before proceeding.

c Incubate the samples for 5 minutes at 70°C in the thermal cycler with the heated lid on.

d Put the plate or strip tube in the magnetic separator at room temperature.

e Wait 1 minute for the solution to clear, then remove and discard the supernatant.

f Repeat step a through step e five more times for a total of 6 washes.

10 After verifying that all wash buffer has been removed, add 25 µl of nuclease-free water to each sample well. Pipette up and down 8 times to resuspend the beads.

Keep the samples on ice until they are used on page 60.

NOTE Captured DNA is retained on the streptavidin beads during the post-capture amplification step.

5 Post-Capture Sample Processing for Multiplexed Sequencing

This chapter describes the steps to amplify, purify, and assess quality and quantity of the captured libraries. Guidelines are provided to prepare the indexed, molecular barcoded samples for multiplexed sequencing, with optional post-capture pooling.

Step 1. Amplify the captured libraries

In this step, the SureSelect-enriched DNA libraries are PCR amplified.

This step uses the components listed in Table 33. Before you begin, thaw the reagents listed below and keep on ice. Remove the AMPure XP beads from cold storage and equilibrate to room temperature for at least 30 minutes in preparation for use on page 61. *Do not freeze the beads at any time.*

Table 33 Reagents for post-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module, Box 2 (Post PCR), –20°C	Pipette up and down 15–20 times	page 60
5× Herculase II Buffer with dNTPs (clear cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module, Box 2 (Post PCR), –20°C	Vortexing	page 60
SureSelect Post-Capture Primer Mix (clear cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module, Box 2 (Post PCR), –20°C	Vortexing	page 60

Prepare one amplification reaction for each target-enriched DNA library pool.

CAUTION To avoid cross-contaminating libraries, set up PCR mixes in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

1 Preprogram a thermal cycler (with heated lid ON) with the program in Table 34. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 34 Post-capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	12–16 (See Table 35 for probe design size-based cycle number recommendations)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Table 35 Post-capture PCR cycle number recommendations

Probe Design Size	Cycles
Probes <0.2 Mb	16 cycles
Probes 0.2–3 Mb	14 cycles
Probes 3–5 Mb	13 cycles
Probes>5 Mb	12 cycles

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 36, on ice. Mix well on a vortex mixer.

Table 36 Preparation of post-capture PCR Reaction mix

Reagent	Volume for 1 amplification reaction	Volume for 6 amplification reactions (includes excess)
Nuclease-free water	13 µl	91 µl
5× Herculase II Buffer with dNTPs (clear cap)	10 µl	70 µl
Herculase II Fusion DNA Polymerase (red cap)	1 µl	7 µl
SureSelect Post-Capture Primer Mix (clear cap)	1 µl	7 µl
Total	**25 µl**	**175 µl**

3 Add 25 µl of the PCR reaction mix prepared in Table 36 to each sample well containing 25 µl of bead-bound target-enriched DNA (prepared on page 56 and held on ice).

4 Mix the PCR reactions well by pipetting up and down until the bead suspension is homogeneous. Avoid splashing samples onto well walls; do not spin the samples at this step.

5 Place the plate or strip tube in the thermal cycler, and resume the thermal cycling program in Table 34.

6 When the PCR amplification program is complete, spin the plate or strip tube briefly. Remove the streptavidin-coated beads by placing the plate or strip tube on the magnetic stand at room temperature. Wait 2 minutes for the solution to clear, then **remove each supernatant (approximately 50 µl) to wells of a fresh plate or strip tube.**

The beads can be discarded at this time.

Step 2. Purify the amplified captured libraries using 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 fresh 70% ethanol per sample, plus excess, for use in step 8.
3. Mix the AMPure XP bead suspension well so that the suspension appears homogeneous and consistent in color.
4. Add 50 µl of the homogeneous AMPure XP bead suspension to each amplified DNA sample (approximately 50 µl) in the PCR plate or strip tube. Mix well by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 3 to 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 disturb the beads while removing the solution.
8. Continue to keep the plate or tubes 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 and step 9 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.
11. Seal the wells with strip caps, then briefly spin 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 25 µl of Low TE buffer (10 mM Tris pH 7.5-8.0, 0.1 mM EDTA) to each sample well.

14 Seal the sample wells with strip caps, then mix well on a vortex mixer and briefly spin to collect the liquid without pelleting the beads.

15 Incubate for 2 minutes at room temperature.

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

17 Transfer the cleared supernatant (approximately 25 µl) to a fresh well. You can discard the beads at this time.

Step 3. Assess sequencing library DNA quantity and quality

Analyze each library using one of the platforms listed in Table 37. Follow the instructions in the linked user guide provided for each assay in Table 37, after reviewing the post-capture library qualification steps below. See Table 38 for expected fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided to illustrate typical results for post-capture libraries prepared from either high-quality or FFPE RNA samples.

Table 37 Post-capture 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	High Sensitivity D1000 ScreenTape	Agilent High Sensitivity D1000 Assay Quick Guide	2 µl
Agilent 2100 Bioanalyzer system	High Sensitivity DNA Kit	Agilent High Sensitivity DNA Kit Guide	1 µl
Agilent 5200, 5300, or 5400 Fragment Analyzer system	HS NGS Fragment Kit (1-6000 bp)	Agilent HS NGS Fragment Kit (1-6000 bp) Kit Guide	2 µl

Table 38 Post-capture library qualification guidelines

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

1 Set up the instrument as instructed in the appropriate user guide (links provided in Table 37).

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 38 for guidelines). Sample TapeStation system electropherograms are shown for libraries prepared from high-quality intact RNA in Figure 4 and from FFPE RNA in Figure 5.

Electropherograms obtained using the other analysis platform options listed in Table 37 are expected to show similar fragment size profiles.

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

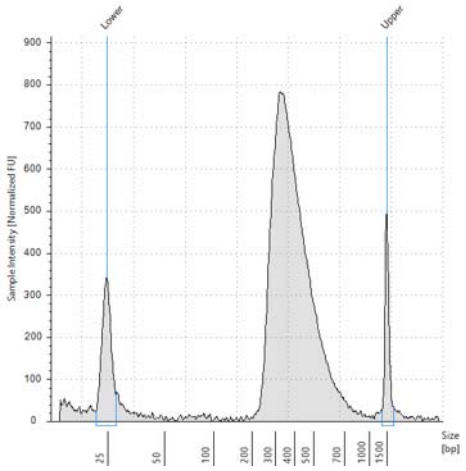


Figure 4 Post-capture library prepared from an intact RNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

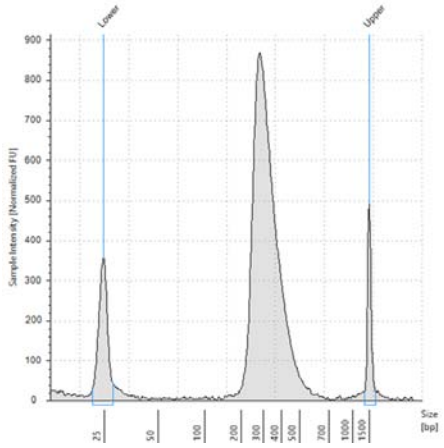


Figure 5 Post-capture library prepared from a typical FFPE RNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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.

Step 4. Optional: Pool samples for multiplexed sequencing

The final captured DNA samples contain pools of either 8 or 16 indexed libraries, based on the Probe Capture Library used and resulting pre-capture pooling strategy. When appropriate for your sequencing platform, the 8-plex or 16-plex samples may be further multiplexed by post-capture pooling.

Determine whether to do post-capture pooling by calculating the number of indexes that can be combined per lane, according to the output specifications of the platform used, together with the amount of sequencing data required for your research design. If doing post-capture pooling, use the guidelines provided below.

If the pre-capture pooled samples will not be further combined in post-capture pools, proceed to page 68.

Combine the pre-capture pooled samples in equimolar amounts in the final post-capture 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 samples, and

C(i) is the initial concentration of each sample

Table 39 shows an example of the amount of 4 pre-capture pooled samples (of different concentrations) and Low TE needed for a final volume of 20 µl at 10 nM DNA.

Table 39 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 to 0.1% v/v and store at -20°C short term.

Step 5. Prepare sequencing samples

The final SureSelect XT HS2 RNA library pool is ready for direct sequencing using standard Illumina paired-end primers and chemistry. Each fragment in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform, as shown in Figure 6.

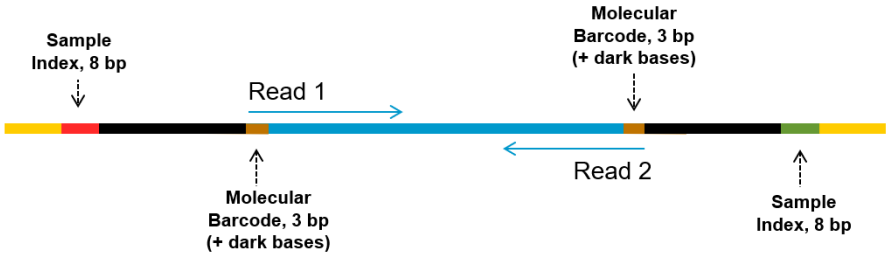


Figure 6 Content of SureSelect XT HS2 sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the dual sample indexes (red and green), dual molecular barcodes (brown) and the library bridge PCR primers (yellow).

Libraries can be sequenced on the Illumina HiSeq, MiSeq, NextSeq, or NovaSeq platform using the run type and chemistry combinations shown in Table 40.

The optimal seeding concentration for SureSelect XT HS2 RNA target-enriched libraries varies according to sequencing platform, run type, and Illumina kit version. See Table 40 for guidelines. Seeding concentration and cluster density may also need to be optimized based on the library cDNA 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 40.

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

Table 40 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	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	300–400 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

Step 6. 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 two sample-level indexes requires an 8-bp index read. For complete index sequence information, see page 81 through page 91.
- For the HiSeq, NextSeq, and NovaSeq platforms, set up the run using the instrument's user interface, following the guidelines on page 71.
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed on page 71 to page 74 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 6 on page 68). Note that unlike DNA, where both strands are present and the MBCs in the strands can be matched to form a duplex consensus read, analysis of single-stranded RNA is limited to consensus generation using the MBC from one strand.
- 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 75 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 75.
- 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 75 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 41. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface and enter the **Cycles** settings in Table 41.

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 41. In the **Custom Primers** section, clear (do **not** select) the checkboxes for all primers (*Read 1, Read 2, Index 1* and *Index 2*).

Table 41 Run settings

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

MiSeq platform sequencing run setup guidelines

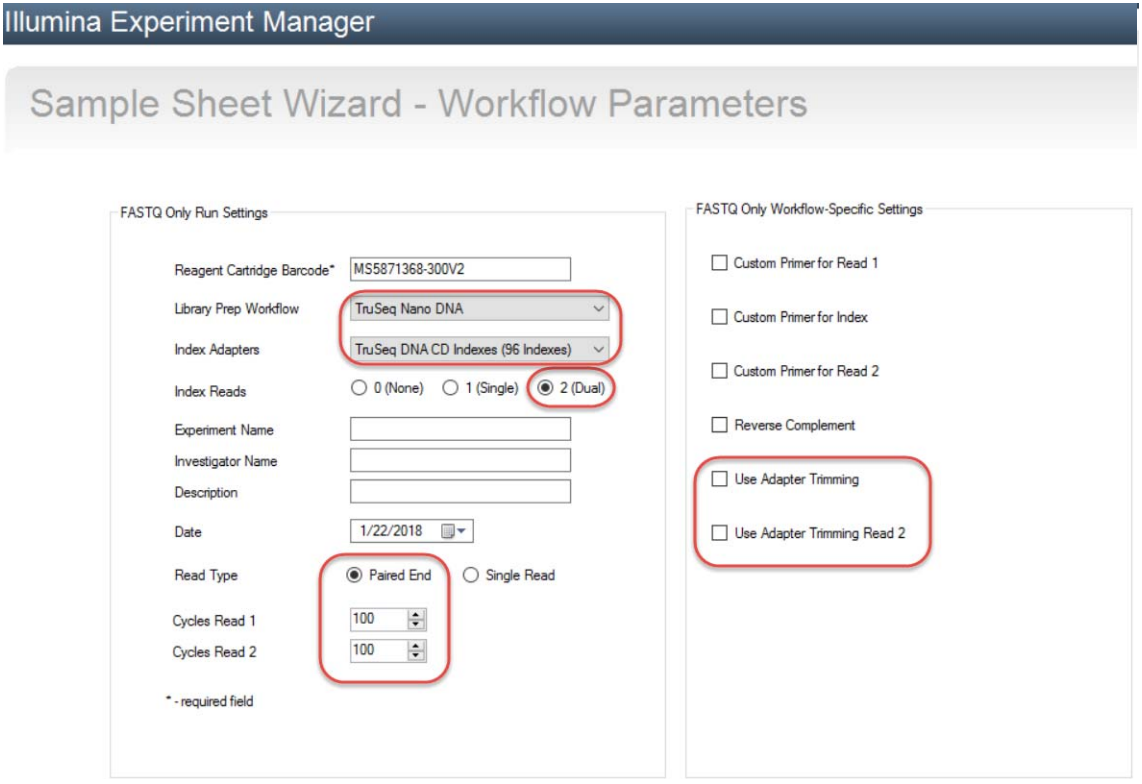
Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below. Once a Sample Sheet has been generated, index sequences need to be manually changed to the SureSelect XT HS2 indexes used for each sample. See Table 48 on page 82 through Table 55 on page 89 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*.

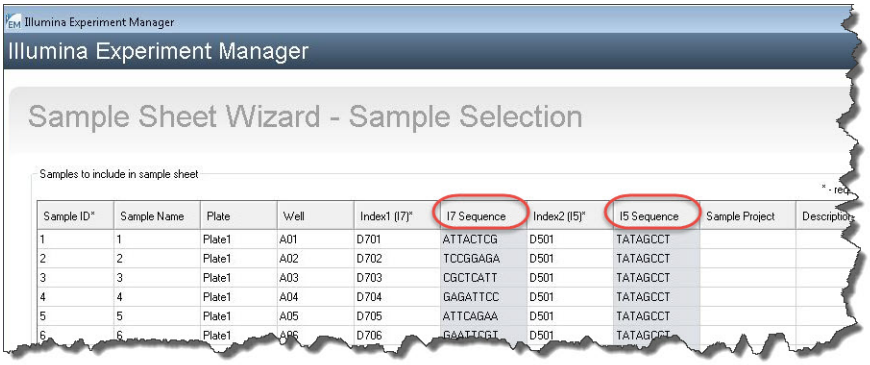
2 On the **Workflow Parameters** screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA**. In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. Make sure to clear both adaptor-trimming 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.



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 Table 48 on page 82 through Table 55 on page 89 for nucleotide sequences of the SureSelect XT HS2 index pairs.
- 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. Note that the P5 indexes are shown in two orientations in Table 48 through Table 55; check the table column headings carefully to locate the P5 sequences appropriate for your sequencing platform.

[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 7 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 and review the AGeNT Best Practices document for processing steps suitable for XT HS2 RNA libraries.

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. The AGeNT trimmer module can also be used to remove the MBCs and properly remove adaptor sequences. Standard adaptor trimmers will fail to remove the MBC sequences from the opposite adaptor (refer to Figure 6), which may affect alignment quality.

The trimmed reads should be aligned using a suitable RNA data alignment tool. Once alignment is complete, the AGeNT LocatIt module may be used in the single-strand consensus mode 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 RNA 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.

6 Reference

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

Kit Contents

The SureSelect XT HS2 RNA Target Enrichment with Pre-capture Pooling protocol uses the kits listed in Table 42. Detailed contents of each of the multi-part component kits listed in Table 42 are shown in Table 43 through Table 47 on the following pages

Table 42 Component Kits

Kit Name (p/n)	Component Kit Name	Component Kit p/n	Storage Condition
SureSelect XT HS2 RNA Library Preparation Kit, 96 Reactions (G9993A through G9993D)	SureSelect cDNA Module (Pre PCR)	5500-0149	–20°C
	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR)	5500-0151	–20°C
	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	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)	–20°C
SureSelect XT HS2 RNA Target Enrichment Kit, 12 Hybs (G9994A)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR)	5191-6689	Room Temperature
	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	5191-6690	–20°C

Table 43 SureSelect cDNA Module (Pre PCR) Content

Kit Component	96 Reaction Kit Format
2X Priming Buffer	tube with purple cap
First Strand Master Mix*	amber tube with amber cap
Second Strand Enzyme Mix	bottle
Second Strand Oligo Mix	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 44 SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR) Content

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

Table 45 SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) Content

Kit Component	96 Reaction Kit Format
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	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)

Table 46 SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 1 (Post PCR) Content

Kit Component	12 Hyb (96 Sample) Kit Format
SureSelect Binding Buffer	bottle
SureSelect Wash Buffer 1	bottle
SureSelect Wash Buffer 2	bottle

Table 47 SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR) Content

Kit Component	12 Hyb (96 Sample) Kit Format
SureSelect Fast Hybridization Buffer	bottle
SureSelect XT HS2 Blocker Mix	tube with blue cap
SureSelect RNase Block	tube with purple cap
SureSelect Post-Capture Primer Mix	tube with clear cap
Herculase II Fusion DNA Polymerase	tube with red cap
5× Herculase II Buffer with dNTPs	tube with clear cap

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 48 through Table 55. See page 70 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 a 96-well plate (see page 90 through page 91 for plate maps). Each well contains a single-use aliquot of a specific pair of forward plus reverse primers.

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 48 SureSelect XT HS2 Index Primer Pairs 1–48, 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
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 49 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 50 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 51 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 52 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 53 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 54 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 55 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

Table 56 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 57 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 58 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 59 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 pre-capture 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 33) 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 pre-capture 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.

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 pre-capture library fragment size is different than expected in electropherograms

- ✔ FFPE RNA pre-capture libraries may have a smaller fragment size distribution due to the presence of fragments in the input RNA that are smaller than the target RNA fragment size.

✔ 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 bead volume recommended for pre-capture purification on page 42.

If low molecular weight adaptor-dimer peak is present in pre-capture 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 to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to the samples analyzed on page 44. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield of pre-capture libraries. If excessive adaptor-dimers are observed, verify that the adaptor ligation protocol is being performed as directed on page 36. In particular, ensure that the Ligation master mix is mixed with the sample prior to adding the 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.

If yield of post-capture libraries is low

✔ PCR cycle number may require optimization. Repeat library preparation and target enrichment for the sample, increasing the post-capture PCR cycle number by 1 to 2 cycles.

✔ The RNA probe capture library used for hybridization may have been compromised. Verify the expiration date on the probe vial or Certificate of Analysis. Adhere to the recommended storage and handling conditions. Ensure that the Capture Library Hybridization Mix is prepared immediately before use, as directed on page 53, and that solutions containing the probe are not held at room temperature for extended periods.

If post-capture 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 bead volume recommended for post-capture purification on page 61.

If low % on-target is observed in library sequencing results

✔ Stringency of post-hybridization washes may have been lower than required. Complete the wash steps as directed, paying special attention to the details of SureSelect Wash Buffer 2 washes listed below:

- SureSelect Wash Buffer 2 is pre-warmed to 70°C (see page 55)
- Samples are maintained at 70°C during washes (see page 56)
- Bead suspensions are mixed thoroughly during washes by pipetting up and down **and** vortexing (see page 56)

✔ Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a vortex and plate spinner or centrifuge in close proximity to thermal cycler to retain the 65°C sample temperature during mixing and transfer steps (step 7 to step 8 on page 53).

If low uniformity of coverage with high AT-dropout is observed in library sequencing results

✔ High AT-dropout may indicate that hybridization conditions are too stringent to obtain the desired level of coverage for AT-rich targets.

- For libraries target-enriched using the SureSelect XT HS Human All Exon V8 Probe and the hybridization program in Table 27 on page 50 (including segment with one-hour incubation at 65°C), repeat target enrichment using the hybridization program in Table 28 on page 50 (without the one-hour incubation at 65°C segment).
- For other probes, repeat target enrichment at lower stringency using a modified thermal cycler program for hybridization, reducing the hybridization temperature in segments 4 and 5 from 65°C to 62.5°C or 60°C (see Table 28 on page 50).

✔ Redesign custom target enrichment probes designed for the XT platform employing SureDesign's XT HS-boosting parameters during design.

Quick Reference Protocol

An abbreviated summary of the protocol steps is provided below for experienced users. Use the complete protocol on page 19 to page 66 until you are familiar with all of the protocol details such as reagent mixing instructions and instrument settings.

Step	Summary of Conditions
	RNA Fragmentation and cDNA Preparation
Prepare and qualify RNA samples	Prepare 10–200 ng total RNA in 10 µl nuclease-free water (high-quality or FFPE samples) For FFPE DNA, qualify integrity as directed on page 22.
Fragment RNA and prime cDNA synthesis	Add 10 µl 2× Priming Buffer to each sample. Place FFPE samples on ice (further fragmentation not required). **For high-quality samples only,** fragment by incubating in thermal cycler: 4 min @ 94°C, 1min @ 4°C, Hold @ 4°C.
Synthesize first-strand cDNA	20 µl primed RNA fragments + 8.5 µl First Strand Master Mix Incubate in thermal cycler: 10 min @ 25°C, 40 min @ 37°C, Hold @ 4°C
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

Step	Summary of Conditions
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 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 40
Purify amplified DNA	50 µl amplified DNA + 50 µl AMPure XP bead suspension Elute DNA in 15 µl nuclease-free H_2O
Quantify and qualify DNA	Analyze quantity and quality using TapeStation, Bioanalyzer, or Fragment Analyzer System
	Hybridization/Capture
Prep DNA in hyb plate	Pool equal amounts of indexed libraries for total of 1.2 µg or 600 ng DNA per pool (see page 48). Adjust volume to 12 µl with nuclease-free H_2O (and using vacuum concentrator where needed).
Program thermal cycler	Input the appropriate thermal cycler program on page 50 and pause program
Run pre-hybridization blocking protocol	12 µl library DNA + 5 µl SureSelect XT HS2 Blocker Mix Run paused thermal cycler program segments 1 through 3; start new pause during segment 3 (1 min @ 65°C)
Prepare Hyb Mix	Prepare 25% RNase Block dilution, then prepare appropriate Capture Library Hyb Mix below: **Probes ≥3 Mb:** 2 µl 25% RNase Block + 5 µl Probe+ 6 µl SureSelect Fast Hybridization Buffer **Probes <3 Mb:** 2 µl 25% RNase Block + 2 µl Probe + 3 µl nuclease-free H_2O + 6 µl SureSelect Fast Hybridization Buffer
Run the hybridization	With cycler paused and samples retained in cycler, add 13 µl Capture Library Hyb Mix to wells Resume the thermal cycler program, completing segments 4 (hybridization) and 5 (65°C hold)
Prepare streptavidin beads	Wash 50 µl Streptavidin T1 beads 3× in 200 µl SureSelect Binding Buffer
Capture hybridized libraries	Add hybridized samples (~30 µl) to washed streptavidin beads (200 µl) Incubate 30 min at RT with vigorous shaking (1400-1900 rpm) During incubation, pre-warm 6 × 200 µl aliquots per sample of SureSelect Wash Buffer 2 to 70°C
Wash captured libraries	Collect streptavidin beads with magnetic stand, discard supernatant Wash beads 1× with 200 µl SureSelect Wash Buffer 1 at RT Wash beads 6× with 200 µl pre-warmed SureSelect Wash Buffer 2 (5 minutes at 70°C per wash) Resuspend washed beads in 25 µl nuclease-free H_2O

Step	Summary of Conditions
	Post-capture amplification
Prepare PCR master mix	Per reaction: 13 µl nuclease-free H_2O+ 10 µl 5× Herculase II Reaction Buffer with dNTPs + 1 µl SureSelect Post-Capture Primer Mix + 1 µl Herculase II Fusion DNA Polymerase Keep on ice
Amplify the bead-bound captured libraries	25 µl DNA bead suspension+ 25 µl PCR master mix Amplify in thermal cycler using conditions on page 59
Purify amplified DNA	Remove streptavidin beads using magnetic stand; retain supernatant 50 µl amplified DNA + 50 µl AMPure XP bead suspension Elute DNA in 25 µl Low TE
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 RNA Reagent Kits to prepare pre-capture pooled NGS libraries for the Illumina platform.

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