

# **SureSelect XT HS2 RNA System**

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

## **Protocol**

**Version C0, June 2022**

**SureSelect platform manufactured with Agilent SurePrint Technology**

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5301 Stevens Creek Blvd  
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## In this Guide...

This guide provides an optimized protocol for preparation of target-enriched Illumina paired-end multiplexed sequencing libraries 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 C0

- Support for SureSelect XT HS Human All Exon V8+UTR Probe. See [Table 2](#) on page 13 for ordering information and see [Table 28](#) on page 49 for the hybridization thermal cycling program recommended for the SureSelect XT HS Human All Exon V8 probes with the SureSelect XT HS2 RNA system.
- Minor update to workflow overview figure on [page 10](#).
- Update to [page 21](#) to clarify requirement for fragmentation of intact control RNA samples.
- Updates to intact input RNA sample qualification details (see [page 24](#)).
- *Note* on SureCycler use removed from [page 25](#).
- Updates to recommended reagent volumes for 24 reaction volume runs in [Table 15](#) on page 33, [Table 17](#) on page 35, [Table 23](#) on page 41, and [Table 37](#) on page 60.
- New AmpPure XP bead purification protocol parameter summary tables for experienced users (see [Table 13](#) on page 28, [Table 19](#) on page 37, [Table 24](#) on page 42, and [Table 38](#) on page 61).
- Update to index equivalence *Note* on [page 39](#) to include plate color.
- Updates to downstream sequencing support information (see [page 70](#) to [page 76](#)). Key updates include support for the new CReaK tool, replacing the LocatIt tool in AGeNT v3.0 (see [page 75](#) to [page 76](#)), and instructions for MBC trimming using BCL Convert software (see *Note* on [page 75](#)).
- Updates to SureSelect XT HS2 Index Primer Pair information on [page 81](#) through [page 89](#) to clarify P5 index sequence orientation usage.
- Update to *Troubleshooting* on [page 95](#) on thermal cycler block configuration requirements for efficient heating of SureSelect Wash Buffer 2.

## What's New in Version B0

- Support for SureSelect XT HS Human All Exon V8 Probe. See [Table 2](#) on page 13 for ordering information and see [Table 28](#) on page 49 for the hybridization thermal cycling program recommended for this probe with the SureSelect XT HS2 RNA system. Also see troubleshooting information on [page 95](#) and updates to the *Quick Reference Protocol* on [page 97](#).
- Update to description of flat strip caps in [Table 6](#) 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 instructions for using index primer pairs provided in 8-well strip tubes in 16 reaction kits. See new *Note* on [page 39](#) and guidance on [page 90](#).
- Updates to “[Hybridization and Capture](#)” instructions. Updates include provision of two separate hybridization thermal cycler programs ([Table 28](#) and [Table 29](#) on page 49), addition of hybridization temperature considerations for probes designed for use with the SureSelect XT system ([Table 29](#) on page 49) and additional minor changes.
- Updates to “[Post-Capture Sample Processing for Multiplexed Sequencing](#)” instructions. Updates include changes to [Table 42](#) on page 69, revised guidance for RNA sequence processing using AGeNT on [page 75](#), and additional minor changes.

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# 1 Before You Begin

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

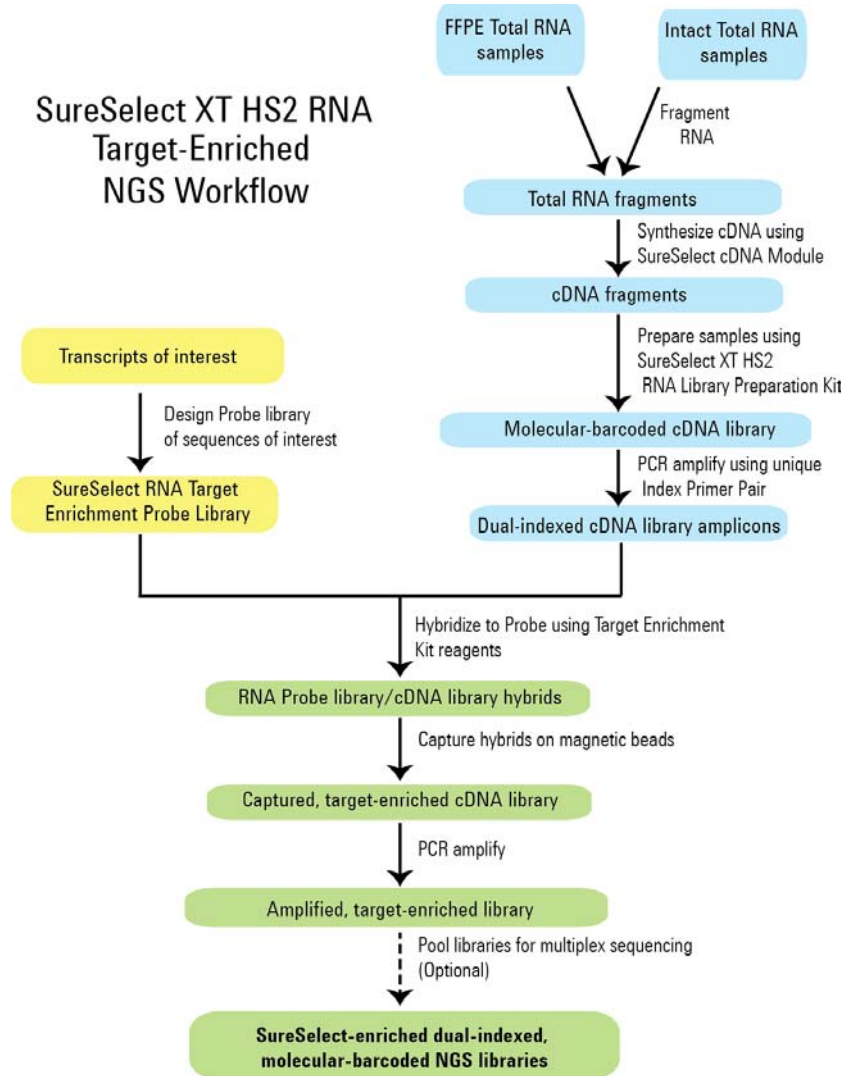
## NOTE

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

Materials required to complete the SureSelect XT HS2 RNA protocol are listed in the tables in this section. Select the preferred SureSelect XT HS2 RNA Reagent Kit format from [Table 1](#), and a target enrichment probe from [Table 2](#). Then refer to [Table 3](#) through [Table 5](#) for additional materials needed to complete the protocols using the selected kit format/RNA sample type.

**Table 1** SureSelect XT HS2 RNA Reagent Kit Varieties

Description	Kit Part Number	
	16 Reaction Kit <sup>*</sup>	96 Reaction Kit <sup>†</sup>
<b>SureSelect XT HS2 RNA Reagent Kit</b>	G9989A (with Index Pairs 1–16)	G9991A (with Index Pairs 1–96) G9991B (with Index Pairs 97–192) G9991C (with Index Pairs 193–288) G9991D (with Index Pairs 289–384)
<b>SureSelect XT HS2 RNA Reagent Kit with AMPure<sup>®</sup> XP/Streptavidin Beads<sup>‡</sup></b>	G9990A (with Index Pairs 1–16)	G9992A (with Index Pairs 1–96) G9992B (with Index Pairs 97–192) G9992C (with Index Pairs 193–288) G9992D (with Index Pairs 289–384)

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

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

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

**Table 2** Compatible Probes

Probe Capture Library	Design Target	16 Reactions	96 Reactions
<b>Pre-designed Probes</b>			
SureSelect XT HS Human All Exon V8	Genome	5191-6873	5191-6874
SureSelect XT HS Human All Exon V8+UTR	Genome	5191-7401	5191-7402
SSel XT HS and XT Low Input Human All Exon V7	Genome	5191-4028	5191-4029
SureSelect XT Clinical Research Exome V2	Genome	5190-9491	5190-9492
SureSelect XT Mouse All Exon	Genome	5190-4641	5190-4642
ClearSeq Comprehensive Cancer XT	Genome	5190-8011	5190-8012
ClearSeq Inherited Disease XT	Genome	5190-7518	5190-7519
ClearSeq RNA Kinome	Transcriptome	5190-4801	5190-4802
<b>Custom Probes*</b>			
SureSelect Custom Tier1 1–499 kb	Custom probes may be designed for either genomic or transcriptomic targets. Please contact the SureSelect support team (see <a href="#">page 2</a> ) or your local representative for assistance with custom probe design and ordering for RNA library target enrichment.		
SureSelect Custom Tier2 0.5–2.9 Mb			
SureSelect Custom Tier3 3–5.9 Mb			
SureSelect Custom Tier4 6–11.9 Mb			
SureSelect Custom Tier5 12–24 Mb			
<b>Pre-designed Probes customized with additional <i>Plus</i> custom content</b>			
SSel XT HS and XT Low Input Human All Exon V7 Plus 1	Genome	Please visit the <a href="#">SureDesign website</a> to design the customized <i>Plus</i> content and obtain ordering information. Contact the SureSelect support team (see <a href="#">page 2</a> ) or your local representative if you need assistance.	
SSel XT HS and XT Low Input Human All Exon V7 Plus 2	Genome		
SureSelect XT Clinical Research Exome V2 Plus 1	Genome		
SureSelect XT Clinical Research Exome V2 Plus 2	Genome		
ClearSeq Comprehensive Cancer Plus XT	Genome		
ClearSeq Inherited Disease Plus XT	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 categories use the same optimized target enrichment protocols detailed in this publication.

## 1 Before You Begin

### Materials Required

**Table 3** Required Reagents

Description	Vendor and Part Number	Notes
1X Low TE Buffer	Thermo Fisher Scientific p/n 12090-015, or equivalent	10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276	—
Nuclease-free Water	Thermo Fisher Scientific p/n AM9930	Water should not be DEPC-treated
AMPure <sup>®</sup> XP Kit	Beckman Coulter Genomics	Separate purchase <b>not</b> required for use with SureSelect XT HS2 RNA Reagent Kits that include SureSelect DNA AMPure <sup>®</sup> XP Beads and SureSelect Streptavidin Beads (Agilent p/n G9990A, G9992A, G9992B, G9992C, or G9992D)
5 ml	p/n A63880	
60 ml	p/n A63881	
450 ml	p/n A63882	
Dynabeads MyOne Streptavidin T1	Thermo Fisher Scientific	
2 ml	p/n 65601	
10 ml	p/n 65602	
50 ml	p/n 65604D	
QPCR Human Reference Total RNA	Agilent p/n 750500	Control input RNA (optional)

**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  $\geq 0.25$  ml per well.

**Table 4** 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
Multichannel pipette	Rainin Pipet-Lite Multi Pipette or equivalent
Single channel pipettes (10-, 20-, 200-, and 1000- $\mu$ l 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.

## 1 Before You Begin

### Materials Required

**Table 5** 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 6** Supplier Information for Optional Materials

Description	Vendor and Part Number	Purpose
Tween 20	Sigma-Aldrich p/n P9416-50ML	Sequencing library storage (see <a href="#">page 67</a> )
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 <a href="#">page 2</a> ) 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.

**1 Before You Begin**  
Optional Materials



## 2 Preparation of Input RNA and Conversion to cDNA

- Step 1A. Prepare and qualify FFPE RNA samples 21
- Step 1B. Prepare and fragment intact RNA samples 24
- Step 2. Synthesize first-strand cDNA 26
- Step 3. Synthesize second-strand cDNA 27
- Step 4. Purify cDNA using AMPure XP beads 28

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.



## 2 Preparation of Input RNA and Conversion to cDNA

The protocol steps in this section use the components listed in [Table 7](#). Thaw and mix each component as directed in [Table 7](#) before use (refer to the *Where Used* column).

**Table 7** 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	<a href="#">page 23</a> (FFPE RNA) <b>OR</b> <a href="#">page 25</a> (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	<a href="#">page 26</a>
Second Strand Enzyme Mix (tube with blue cap or bottle)	SureSelect cDNA Module (Pre PCR), -20°C	Thaw on ice then keep on ice	Vortexing	<a href="#">page 27</a>
Second Strand Oligo Mix (tube with yellow cap)	SureSelect cDNA Module (Pre PCR), -20°C	Thaw on ice then keep on ice	Vortexing	<a href="#">page 27</a>

\* 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.

### NOTE

AMPure XP beads must be removed from cold storage and equilibrated to room temperature for at least 30 minutes in preparation for use on [page 28](#). *Do not freeze the beads at any time.*

## 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  $\mu$ l 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. The control RNA sample must be fragmented as described on [page 24](#) before processing in parallel with the FFPE samples for cDNA synthesis and later protocol steps.

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 Preparation of Input RNA and Conversion to cDNA

### Step 1A. Prepare and qualify FFPE RNA samples

- 2 Examine the starting size distribution of RNA in the sample using one of the RNA qualification systems described in [Table 8](#). 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 8](#). RNA molecules must be >200 nt for efficient conversion to cDNA library.

**Table 8** 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 9](#).

**Table 9** 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  $\mu$ l 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.

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.

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 [step 5](#) below 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 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 Verify the RNA integrity in the sample using one of the qualification systems described in [Table 8](#) on page 22. Select the specific assay appropriate for your sample based on the RNA concentration determined in [step 1](#).

Determine the DV200 (percentage of RNA in the sample that is >200 nt) using the analysis mode described in [Table 8](#). RNA molecules must be >200 nt for efficient conversion to cDNA library fragments. Samples with DV200<50% may not be suitable for use in RNA target enrichment applications.

- 3** Preprogram a thermal cycler with the program in [Table 10](#). Immediately pause the program, and keep paused until samples are loaded in [step 7](#).

**Table 10** 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.

- 4** 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.
- 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.
- 7** Place the samples in the thermal cycler, and resume the thermal cycling program in [Table 10](#) for RNA fragmentation.
- 8** Once the thermal cycler program in [Table 10](#) reaches the 4°C Hold step, transfer the fragmented RNA sample plate or strip tube from the thermal cycler to ice or a cold block. Proceed immediately to [“Step 2. Synthesize first-strand cDNA”](#) on page 26.

## 2 Preparation of Input RNA and Conversion to cDNA

### Step 2. Synthesize first-strand cDNA

## 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 11](#). Immediately pause the program, and keep paused until samples are loaded in [step 5](#).

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

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

\* Use a reaction volume setting of 28  $\mu$ l, 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  $\mu$ l of First Strand Master Mix to each RNA sample well.
- 4 Mix well by pipetting up and down 15–20 times or seal the wells and vortex at high speed for 5–10 seconds. Spin briefly to collect the liquid.
- 5 Place the samples in the thermal cycler, and resume the program in [Table 11](#).

### Step 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 11](#) begins the 4°C hold step, transfer the samples to ice.
- 2 Preprogram the thermal cycler with the program in [Table 12](#). Immediately pause the program, and keep paused until samples are loaded in [step 7](#).

**Table 12** Thermal cycler program for second-strand synthesis \*

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

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

- 3 Vortex the thawed vials of Second Strand Enzyme Mix (blue cap or 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 12](#).

#### NOTE

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

## 2 Preparation of Input RNA and Conversion to cDNA

### Step 4. Purify cDNA using AMPure XP beads

#### Step 4. Purify cDNA using AMPure XP beads

In this step, the cDNA samples are purified using AmpPure XP Beads. Critical purification protocol parameters are summarized for experienced users in [Table 13](#).

**Table 13** AMPure XP bead cleanup parameters after cDNA synthesis

Parameter	Value
Volume of RT AMPure XP bead suspension added to each sample well	105 $\mu$ l
Final elution solvent and volume	52 $\mu$ l nuclease-free water
Amount of eluted sample transferred to fresh well	50 $\mu$ l

- 1 Verify that the AMPure XP beads have been held at room temperature for at least 30 minutes before use.
- 2 Prepare 400  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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.

## **2 Preparation of Input RNA and Conversion to cDNA**

### **Step 4. Purify cDNA using AMPure XP beads**



## 3 Library Preparation

- Step 1. Prepare the Ligation master mix 33
- Step 2. Repair and dA-Tail the cDNA 3' ends 34
- Step 3. Ligate the molecular-barcoded adaptor 36
- Step 4. Purify the sample using AMPure XP beads 37
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- Step 6. Purify the amplified library with AMPure XP beads 42
- Step 7. Assess quality and quantity 44

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).

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



### 3 Library Preparation

**Table 14** Reagents thawed before use in protocol

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
Ligation Buffer (purple cap or bottle)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), -20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	<a href="#">page 33</a>
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	<a href="#">page 33</a>
End Repair-A Tailing Buffer (yellow cap or bottle)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), -20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	<a href="#">page 35</a>
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	<a href="#">page 35</a>
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	<a href="#">page 36</a>

#### NOTE

AMPure XP beads must be removed from cold storage and equilibrated to room temperature for at least 30 minutes in preparation for use on [page 37](#). *Do not freeze the beads at any time.*

## 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 8 reactions* (includes excess)	Volume for 24 reactions† (includes excess)
Ligation Buffer (purple cap or bottle)	23 µl	207 µl	598 µl
T4 DNA Ligase (blue cap)	2 µl	18 µl	52 µl
<b>Total</b>	<b>25 µl</b>	<b>225 µl</b>	<b>650 µl</b>

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

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

### 3 Library Preparation

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

## 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  $\mu$ l, 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 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
End Repair-A Tailing Buffer (yellow cap or bottle)	16 $\mu$ l	144 $\mu$ l	416 $\mu$ l
End Repair-A Tailing Enzyme Mix (orange cap)	4 $\mu$ l	36 $\mu$ l	104 $\mu$ l
<b>Total</b>	<b>20 <math>\mu</math>l</b>	<b>180 <math>\mu</math>l</b>	<b>520 <math>\mu</math>l</b>

- 4 Add 20  $\mu$ l of the End Repair/dA-Tailing master mix to each sample well containing approximately 50  $\mu$ l of purified cDNA sample. Mix by pipetting up and down 15–20 times using a pipette set to 50  $\mu$ l 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](#).

### 3 Library Preparation

#### Step 3. Ligate the molecular-barcoded adaptor

## 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 (MBC) sequences are incorporated into both ends of each library DNA fragment at this step.

## Step 4. Purify the sample using AMPure XP beads

In this step, the cDNA libraries are purified using AmpPure XP Beads. Critical purification protocol parameters are summarized for experienced users in [Table 19](#).

**Table 19** AMPure XP bead cleanup parameters after adaptor ligation

Parameter	Value
Volume of RT AMPure XP bead suspension added to each sample well	80 $\mu$ l
Final elution solvent and volume	35 $\mu$ l nuclease-free water
Amount of eluted sample transferred to fresh well	Approximately 34 $\mu$ l

- 1 Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use.
- 2 Prepare 400  $\mu$ l 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  $\mu$ l of homogeneous AMPure XP beads to each cDNA library sample (approximately 100  $\mu$ l) 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  $\mu$ l 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.

### 3 Library Preparation

#### Step 4. Purify the sample using AMPure XP beads

- 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 20](#). Before you begin, thaw the reagents listed below and keep on ice.

**Table 20** 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	<a href="#">page 41</a>
5× Herculase II Buffer with dNTPs (clear cap)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	<a href="#">page 41</a>
SureSelect XT HS2 Index Primer Pairs	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR),* –20°C	Vortexing	<a href="#">page 41</a>

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

### NOTE

AMPure XP beads must be removed from cold storage and equilibrated to room temperature for at least 30 minutes in preparation for use on [page 42](#).

**1** Determine the appropriate index pair assignment for each sample. See [Table 51](#) on page 82 through [Table 58](#) 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.

### NOTE

Index pairs 1-16 provided in strip tubes in 16-reaction kits are equivalent to index pairs 1-16 provided in orange plates in 96-reaction kits; do not use the same-numbered index pair from a strip tube and a plate for multiplexed samples.

When using index pairs provided in strip tubes in [step 5](#) on [page 41](#), verify the strip tube orientation using the numeral **1** or **9** etched adjacent to the lowest-numbered index and the strip barcode adjacent to the highest-numbered index. Pierce the foil seal of the appropriate well with a pipette tip just before pipetting the solution.

### 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.

### 3 Library Preparation

#### Step 5. Amplify the adaptor-ligated cDNA library

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

**Table 21** Pre-Capture PCR Thermal Cycler Program\*

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10–14 (See <a href="#">Table 22</a> 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  $\mu$ l, if required for thermal cycler set up.

**Table 22** 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

#### NOTE

Libraries prepared from high-quality (intact) input RNA must be placed in a separate strip tube or PCR plate from FFPE RNA input libraries since these sample types are processed using different PCR conditions.

**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 23](#), on ice. Mix well on a vortex mixer.

**Table 23** Preparation of Pre-Capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
5× Herculase II Buffer with dNTPs (clear cap)	10 µl	90 µl	260 µl
Herculase II Fusion DNA Polymerase (red cap)	1 µl	9 µl	26 µl
<b>Total</b>	<b>11 µl</b>	<b>99 µl</b>	<b>286 µl</b>

- 4 Add 11 µl of the PCR reaction mixture prepared in [Table 23](#) to each purified DNA library sample (34 µl) in the PCR plate wells.
- 5 Add 5 µl of the appropriate SureSelect XT HS2 Index Primer Pair to each reaction.  
Cap the wells then vortex at high speed for 5 seconds. Spin the plate or strip tube briefly to collect the liquid and release any bubbles.
- 6 Before adding the samples to the thermal cycler, resume the thermal cycling program in [Table 21](#) to bring the temperature of the thermal block to 98°C. Once the cycler has reached 98°C, 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.

### 3 Library Preparation

#### Step 6. Purify the amplified library with AMPure XP beads

## Step 6. Purify the amplified library with AMPure XP beads

In this step, the amplified DNA libraries are purified using AmpPure XP Beads. Critical purification protocol parameters are summarized for experienced users in [Table 24](#).

**Table 24** AMPure XP bead cleanup parameters after pre-capture PCR

Parameter	Value
Volume of RT AMPure XP bead suspension added to each sample well	50 $\mu$ l
Final elution solvent and volume	15 $\mu$ l nuclease-free water
Amount of eluted sample transferred to fresh well	Approximately 15 $\mu$ l

- 1 Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use.
- 2 Prepare 400  $\mu$ l 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  $\mu$ l of homogeneous AMPure XP beads to each 50- $\mu$ l 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  $\mu$ l 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.

## Step 6. Purify the amplified library with AMPure XP beads

- 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 25](#). Follow the instructions in the linked user guide provided for each assay in [Table 25](#), 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 26](#) 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 25** 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	<a href="#">Agilent D1000 Assay Quick Guide</a>	1 µl
Agilent 2100 Bioanalyzer system	DNA 1000 Kit	<a href="#">Agilent DNA 1000 Kit Guide</a>	1 µl
Agilent 5200, 5300, or 5400 Fragment Analyzer system	NGS Fragment Kit (1-6000 bp)	<a href="#">Agilent NGS Fragment Kit (1-6000 bp) Kit Guide</a>	2 µl

**Table 26** Pre-capture library qualification guidelines

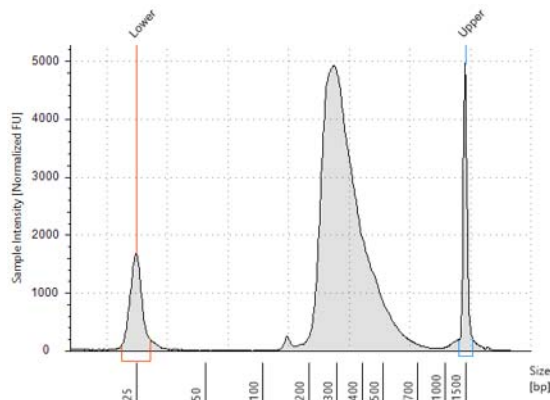
Input RNA type	Expected library DNA fragment size peak position	NGS read lengths supported
High-quality RNA or FFPE RNA	200 to 700 bp	2 ×100 reads or 2 ×150 reads

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 94](#) for additional considerations.

- 1 Set up the instrument as instructed in the appropriate user guide (links provided in [Table 25](#)).
- 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 26](#) 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 25](#) are expected to show similar fragment size profiles.

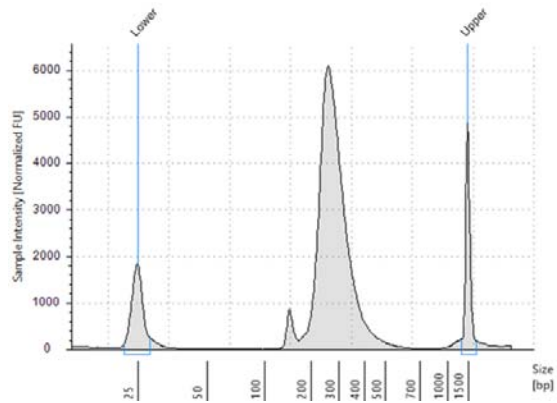
- 4 Determine the concentration of the library DNA by integrating under the peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.



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

### 3 Library Preparation

#### Step 7. Assess quality and quantity



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



## 4 Hybridization and Capture

- Step 1. Hybridize cDNA libraries to the probe 48
- Step 2. Prepare streptavidin-coated magnetic beads 53
- Step 3. Capture the hybridized DNA using streptavidin-coated beads 54

This chapter describes the steps to hybridize the prepared cDNA libraries with a target-specific probe. After hybridization, the targeted molecules are captured on streptavidin-coated beads. Each cDNA library sample is hybridized and captured individually.

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 50](#).

### CAUTION

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



## 4 Hybridization and Capture

### Step 1. Hybridize cDNA libraries to the probe

## Step 1. Hybridize cDNA libraries to the probe

In this step, the prepared cDNA libraries are hybridized to a target-specific probe. For each RNA sample library prepared, do one hybridization and capture. Do not pool samples at this stage.

The hybridization reaction requires 200 ng of prepared cDNA library in a volume of 12  $\mu$ l.

This step uses the components listed in [Table 27](#). Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

**Table 27** 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), $-20^{\circ}\text{C}$	Thaw on ice	<a href="#">page 50</a>
SureSelect RNase Block (purple cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), $-20^{\circ}\text{C}$	Thaw on ice	<a href="#">page 51</a>
SureSelect Fast Hybridization Buffer (bottle)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), $-20^{\circ}\text{C}$	Thaw and keep at Room Temperature	<a href="#">page 51</a>
Probe	$-80^{\circ}\text{C}$	Thaw on ice	<a href="#">page 51</a>

- 1 Preprogram a thermal cycler (with heated lid ON) with the program in [Table 28](#) for the SureSelect XT HS Human All Exon V8 Probes or in [Table 29](#) for all other probes. Immediately pause the program, and keep paused until samples are loaded in [step 4](#) on [page 50](#).

**Table 28** Hybridization program for SureSelect XT HS Human All Exon V8 Probes (V8 or V8+UTR)\*

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 <a href="#">step 7</a> on <a href="#">page 52</a> )
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 <a href="#">page 54</a>

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

**Table 29** 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 <a href="#">step 7</a> on <a href="#">page 52</a> )
4	60	65°C <sup>†</sup>	1 minute
		37°C	3 seconds
5	1	65°C <sup>†</sup>	Hold briefly until ready to begin capture steps on <a href="#">page 54</a>

\* 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 XT platform, including SureSelect XT Human All Exon V6 (62.5°C), SureSelect XT Clinical Research Exome V2 (62.5°C) and custom probes originally designed for use with SureSelect XT system (60°C–65°C).

## 4 Hybridization and Capture

### Step 1. Hybridize cDNA libraries to the probe

#### NOTE

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

- In the final segment of the thermal cycler program ([Table 28](#) or [Table 29](#)), 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 53](#) on day 2, after the overnight hold.

- 2 Place 200 ng of each prepared cDNA library sample into the well of a fresh hybridization plate or strip tube and then bring the final volume in each well to 12 µl using nuclease-free water.
- 3 To each DNA library sample well, add 5 µl of SureSelect XT HS2 Blocker Mix (blue cap). Seal 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.

#### CAUTION

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

- 4 Transfer the sealed sample plate or strip to the thermal cycler and resume the thermal cycling program ([Table 28](#) or [Table 29](#) on page 49), 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 7](#) on [page 52](#).**

During Segments 1 and 2 of the thermal cycling program, begin preparing the additional hybridization reagents as described in [step 5](#) and [step 6](#) below. If needed, you can finish these preparation steps after pausing the thermal cycler in Segment 3.

- 5 Prepare a 25% solution of SureSelect RNase Block (1 part RNase Block to 3 parts water), according to [Table 30](#). Prepare the amount required for the number of hybridization reactions in the run, plus excess. Mix well and keep on ice.

**Table 30** Preparation of RNase Block solution

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
SureSelect RNase Block	0.5 µl	4.5 µl	12.5 µl
Nuclease-free water	1.5 µl	13.5 µl	37.5 µl
<b>Total</b>	<b>2 µl</b>	<b>18 µl</b>	<b>50 µl</b>

**NOTE**

Prepare the mixture described in [step 6](#), 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 7](#) on [page 52](#). Do not keep solutions containing the probe at room temperature for extended periods.

- 6 Prepare the Probe Hybridization Mix appropriate for your probe design size. Use [Table 31](#) for probes  $\geq 3$  Mb or [Table 32](#) 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 7](#).

**Table 31** Preparation of Probe Hybridization Mix for probes  $\geq 3$  Mb

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
25% RNase Block solution (from <a href="#">step 5</a> )	2 µl	18 µl	50 µl
Probe (with design $\geq 3$ Mb)	5 µl	45 µl	125 µl
SureSelect Fast Hybridization Buffer	6 µl	54 µl	150 µl
<b>Total</b>	<b>13 µl</b>	<b>117 µl</b>	<b>325 µl</b>

## 4 Hybridization and Capture

### Step 1. Hybridize cDNA libraries to the probe

**Table 32** Preparation of Probe Hybridization Mix for **probes <3 Mb**

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
25% RNase Block solution (from <a href="#">step 5</a> )	2 µl	18 µl	50 µl
Probe (with design <3 Mb)	2 µl	18 µl	50 µl
SureSelect Fast Hybridization Buffer	6 µl	54 µl	150 µl
Nuclease-free water	3 µl	27 µl	75 µl
<b>Total</b>	<b>13 µl</b>	<b>117 µl</b>	<b>325 µl</b>

- 7 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 6](#) 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.

- 8 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.
- 9 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 2. Prepare streptavidin-coated magnetic beads

The remaining hybridization capture steps use the reagents in [Table 33](#).

### NOTE

If performing same-day hybridization and capture, begin the bead preparation steps below approximately one hour after starting hybridization in [step 9](#) on [page 52](#). 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 54](#).

**Table 33** Reagents for Capture

Kit Component	Storage Location	Where Used
SureSelect Binding Buffer	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	<a href="#">page 53</a>
SureSelect Wash Buffer 1	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	<a href="#">page 54</a>
SureSelect Wash Buffer 2	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	<a href="#">page 54</a>
SureSelect Streptavidin Beads <b>OR</b> Dynabeads MyOne Streptavidin T1 Beads	4°C	<a href="#">page 53</a>

- 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 3. 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 28](#) or [Table 29](#) on page 49), transfer the samples to room temperature.
- 2 Immediately transfer the entire volume (approximately 30  $\mu$ l) of each hybridization mixture to wells containing 200  $\mu$ l 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- $\mu$ l aliquots of Wash Buffer 2 in wells of a fresh 96-well plate or strip tubes. Aliquot 6 wells of buffer for each sample 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  $\mu$ l 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.

## Step 3. Capture the hybridized DNA using streptavidin-coated beads

**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.

## **4 Hybridization and Capture**

**Step 3. Capture the hybridized DNA using streptavidin-coated beads**



## 5 Post-Capture Sample Processing for Multiplexed Sequencing

- Step 1. Amplify the captured libraries 58
- Step 2. Purify the amplified captured libraries using AMPure XP beads 61
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- Step 5. Prepare sequencing samples 68
- Step 6. Do the sequencing run and analyze the data 70
- Sequence analysis resources 75

This chapter describes the steps to amplify, purify, and assess quality and quantity of the captured libraries. Sample pooling instructions are provided to prepare the indexed, molecular barcoded samples for multiplexed sequencing.



## 5 Post-Capture Sample Processing for Multiplexed Sequencing

### Step 1. Amplify the captured libraries

## 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 34](#). Before you begin, thaw the reagents listed below and keep on ice.

**Table 34** 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	<a href="#">page 60</a>
5× Herculase II Buffer with dNTPs (clear cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module, Box 2 (Post PCR), -20°C	Vortexing	<a href="#">page 60</a>
SureSelect Post-Capture Primer Mix (clear cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module, Box 2 (Post PCR), -20°C	Vortexing	<a href="#">page 60</a>

#### NOTE

AMPure XP beads must be removed from cold storage and equilibrated to room temperature for at least 30 minutes in preparation for use on [page 61](#).

Prepare one amplification reaction for each DNA library.

#### 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 35](#). Immediately pause the program, and keep paused until samples are loaded in [step 5](#).

**Table 35** Post-capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	12–16 (See <a href="#">Table 36</a> 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 36** 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

## 5 Post-Capture Sample Processing for Multiplexed Sequencing

### Step 1. Amplify the captured libraries

- 2 Prepare the appropriate volume of PCR reaction mix, as described in [Table 37](#), on ice. Mix well on a vortex mixer.

**Table 37** Preparation of post-capture PCR Reaction mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
Nuclease-free water	13 $\mu$ l	117 $\mu$ l	338 $\mu$ l
5 $\times$ Herculase II Buffer with dNTPs (clear cap)	10 $\mu$ l	90 $\mu$ l	260 $\mu$ l
Herculase II Fusion DNA Polymerase (red cap)	1 $\mu$ l	9 $\mu$ l	26 $\mu$ l
SureSelect Post-Capture Primer Mix (clear cap)	1 $\mu$ l	9 $\mu$ l	26 $\mu$ l
<b>Total</b>	<b>25 <math>\mu</math>l</b>	<b>225 <math>\mu</math>l</b>	<b>650 <math>\mu</math>l</b>

- 3 Add 25  $\mu$ l of the PCR reaction mix prepared in [Table 37](#) to each sample well containing 25  $\mu$ l of bead-bound target-enriched DNA (prepared on [page 55](#) 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 35](#).
- 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 **transfer each supernatant (approximately 50  $\mu$ l) 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

In this step, the amplified enriched DNA libraries are purified using AmpPure XP Beads. Critical purification protocol parameters are summarized for experienced users in [Table 38](#).

**Table 38** AMPure XP bead cleanup parameters after post-capture PCR

Parameter	Value
Volume of RT AMPure XP bead suspension added to each sample well	50 $\mu$ l
Final elution solvent and volume	25 $\mu$ l Low TE Buffer
Amount of eluted sample transferred to fresh well	Approximately 25 $\mu$ l

- 1 Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use.
- 2 Prepare 400  $\mu$ l 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  $\mu$ l of the homogeneous AMPure XP bead suspension to each amplified DNA sample (approximately 50  $\mu$ l) 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  $\mu$ l of freshly-prepared 70% ethanol in each sample well.

## 5 Post-Capture Sample Processing for Multiplexed Sequencing

### Step 2. Purify the amplified captured libraries using AMPure XP beads

- 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 39](#). Follow the instructions in the linked user guide provided for each assay in [Table 39](#), after reviewing the post-capture library qualification steps below. See [Table 40](#) 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 39** 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	<a href="#">Agilent High Sensitivity D1000 Assay Quick Guide</a>	2 µl
Agilent 2100 Bioanalyzer system	High Sensitivity DNA Kit	<a href="#">Agilent High Sensitivity DNA Kit Guide</a>	1 µl
Agilent 5200, 5300, or 5400 Fragment Analyzer system	HS NGS Fragment Kit (1-6000 bp)	<a href="#">Agilent HS NGS Fragment Kit (1-6000 bp) Kit Guide</a>	2 µl

**Table 40** 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 39](#)).
- 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.

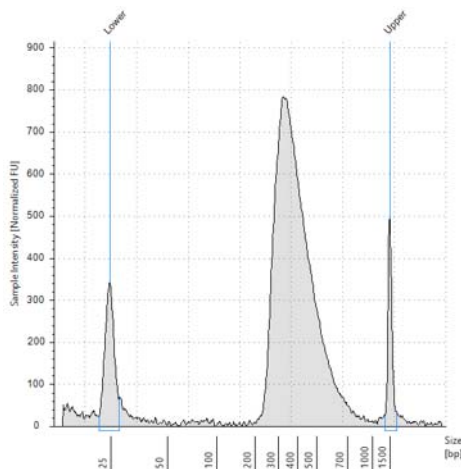
## 5 Post-Capture Sample Processing for Multiplexed Sequencing

### Step 3. Assess sequencing library DNA quantity and quality

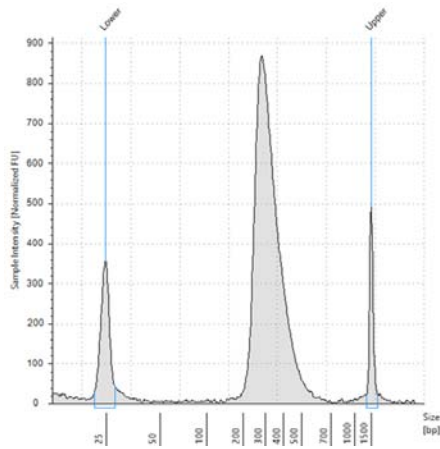
- 3 Verify that the electropherogram shows the expected DNA fragment size peak position (see Table 40 for guidelines). Sample TapeStation system electropherograms are shown for libraries prepared from high-quality intact RNA in Figure 4 and from FFPE RNA in Figure 5.

Electropherograms obtained using the other analysis platform options listed in Table 39 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. Pool samples for multiplexed sequencing

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

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

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

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

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

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

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

$\#$  is the number of indexes, and

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

Table 41 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20  $\mu$ l at 10 nM DNA.

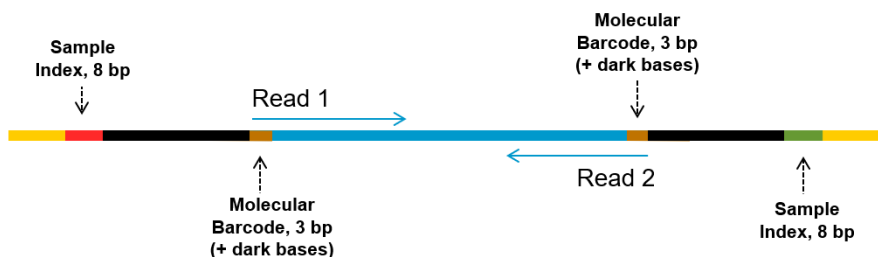
**Table 41** Example of volume calculation for total volume of 20  $\mu$ l at 10 nM concentration

Component	V(f)	C(i)	C(f)	#	Volume to use ( $\mu$ l)
Sample 1	20 $\mu$ l	20 nM	10 nM	4	2.5
Sample 2	20 $\mu$ l	10 nM	10 nM	4	5
Sample 3	20 $\mu$ l	17 nM	10 nM	4	2.9
Sample 4	20 $\mu$ l	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 42](#).

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 42](#) 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 42](#).

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

**Table 42** Illumina Kit Configuration Selection Guidelines

<b>Platform</b>	<b>Run Type</b>	<b>Read Length</b>	<b>SBS Kit Configuration</b>	<b>Chemistry</b>	<b>Seeding Concentration</b>
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 user guide sections with additional details.

- Each of the two sample-level indexes requires an 8-bp index read. For complete index sequence information, see [page 81](#) through [page 92](#).
- 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.
- 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. Adaptors are trimmed in later processing steps as described below to ensure proper processing of the degenerate MBCs in the adaptor sequences.
- Demultiplex using Illumina's bcl2fastq, BCL Convert or DRAGEN software to generate paired end reads based on the dual indexes and remove sequences with incorrectly paired P5 and P7 indexes. Do not use the MBC/UMI trimming options in Illumina's demultiplexing software if using Agilent's Genomics NextGen Toolkit (AGeNT) or SureCall software to process your FASTQ files.
- Before aligning reads to reference sequences, Illumina adaptor sequences should be trimmed from the reads using the AGeNT Trimmer module, which properly accounts for the degenerate MBCs in the adaptor sequence. See [page 75](#) for more information.
- 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. Agilent recommends using AGeNT for 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](#).

## HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface, using the settings shown in [Table 43](#). For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface and enter the **Cycles** settings in [Table 43](#).

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

**Table 43** Run settings

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

## MiSeq platform sequencing run setup guidelines

Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below. Once a Sample Sheet has been generated, index sequences need to be manually changed to the SureSelect XT HS2 indexes used for each sample. See [Table 51](#) on page 82 through [Table 58](#) 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*.

## 5 Post-Capture Sample Processing for Multiplexed Sequencing

### Step 6. Do the sequencing run and analyze the data

- 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**.

## Illumina Experiment Manager

### Sample Sheet Wizard - Workflow Parameters

FASTQ Only Run Settings

Reagent Cartridge Barcode\* MS5871368-300V2

Library Prep Workflow TruSeq Nano DNA

Index Adapters TruSeq DNA CD Indexes (96 Indexes)

Index Reads  0 (None)  1 (Single)  2 (Dual)

Experiment Name

Investigator Name

Description

Date 1/22/2018

Read Type  Paired End  Single Read

Cycles Read 1 100

Cycles Read 2 100

\* - required field

FASTQ Only Workflow-Specific Settings

Custom Primer for Read 1

Custom Primer for Index

Custom Primer for Read 2

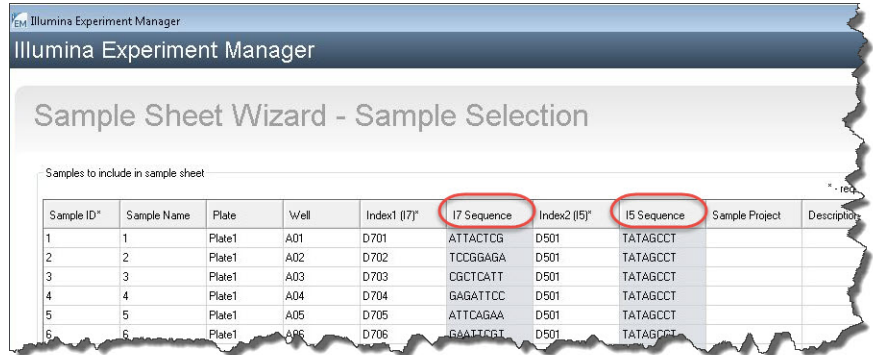
Reverse Complement

Use Adapter Trimming

Use Adapter Trimming Read 2

- 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.

## 5 Post-Capture Sample Processing for Multiplexed Sequencing

### Step 6. Do the sequencing run and analyze the data

#### 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 51](#) on page 82 through [Table 58](#) 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 51](#) through [Table 58](#); 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	3/20/2013							
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 Illumina's bcl2fastq, BCL Convert or DRAGEN 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](http://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 masking or trimming before proceeding to downstream analysis. If demultiplexing using bcl2fastq, MBCs may be masked by including the base mask **N5Y\*,I8,I8,N5Y\*** (where \* is replaced with the actual read length, matching the read length value in the RunInfo.xml file). If demultiplexing using BCL Convert, MBCs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y\*;I8;I8;N5Y\*** (where \* is replaced with read length after trimming, e.g., use N5Y145;I8;I8;N5Y145 for 2x150 NGS).

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 while trimming 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 CReaK (Consensus Read Kit) tool can 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 gene expression and variant discovery.

## 5 Post-Capture Sample Processing for Multiplexed Sequencing

### Sequence analysis resources

#### NOTE

CReaK is a deduplication tool introduced in AGeNT version 3.0, replacing the AGeNT LocatIt tool. Please visit the [AGeNT page at www.agilent.com](http://www.agilent.com) and review the FAQs for a detailed comparison of LocatIt and CReaK. LocatIt remains available for backward compatibility but CReaK is the recommended tool.

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#### 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

Kit Contents	78
SureSelect XT HS2 Index Primer Pair Information	81
Troubleshooting Guide	93
Quick Reference Protocol	96

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



## Kit Contents

SureSelect XT HS2 RNA Reagent Kits include the component kits listed in Table 44. Detailed contents of each of the multi-part component kits listed in Table 44 are shown in Table 45 through Table 49 on the following pages.

**Table 44** Component Kits

Component Kit Name	Storage Condition	Component Kit Part Number	
		16 Reaction Kits	96 Reaction Kits
<b>Standard Component Modules</b>			
SureSelect cDNA Module (Pre PCR)	-20°C	5500-0148	5500-0149
SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR)	-20°C	5500-0150	5500-0151
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	-20°C	5191-5687 (Index Pairs 1–16)	5191-5688 (Index Pairs 1–96), 5191-5689 (Index Pairs 97–192), 5191-5690 (Index Pairs 193–288), OR 5191-5691 (Index Pairs 289–384)
SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	Room Temperature	5190-9685	5190-9687
SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module, Box 2 (Post PCR)	-20°C	5191-6686	5191-6688
<b>Optional Component Modules</b>			
SureSelect RNA AMPure® XP Beads	+4°C	5191-6670*	5191-6671†
SureSelect Streptavidin Beads	+4°C	5191-5741*	5191-5742†

\* Provided only with 16-Reaction Reagent Kit part number G9990A.

† Provided only with 96-Reaction Reagent Kit part numbers G9992A, G9992B, G9992C, G9992D.

The contents of each of the multi-part component kits listed in [Table 44](#) are described in the tables below.

**Table 45** SureSelect cDNA Module (Pre PCR) Content

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

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

**Table 46** SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR) Content

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

**Table 47** SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) Content

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

## 6 Reference

### Kit Contents

**Table 48** SureSelect Target Enrichment Kit, ILM Hyb Module Box 1 (Post PCR) Content

Kit Component	16 Reaction Kit Format	96 Reaction Kit Format
SureSelect Binding Buffer	bottle	bottle
SureSelect Wash Buffer 1	bottle	bottle
SureSelect Wash Buffer 2	bottle	bottle

**Table 49** SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR) Content

Kit Component	16 Reaction Kit Format	96 Reaction Kit Format
SureSelect Fast Hybridization Buffer	bottle	bottle
SureSelect XT HS2 Blocker Mix	tube with blue cap	tube with blue cap
SureSelect RNase Block	tube with purple cap	tube with purple cap
SureSelect Post-Capture Primer Mix	tube with clear cap	tube with clear cap
Herculase II Fusion DNA Polymerase	tube with red cap	tube with red cap
5× Herculase II Buffer with dNTPs	tube with clear cap	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 P7 or P5 index, resulting in dual-indexed NGS libraries. One primer pair is provided in each well of 8-well strip tubes (16 reaction kits; see [Figure 8](#) on page 90 for a map) or of 96-well plates (96 reaction kits; see [page 91](#) through [page 92](#) for plate maps). Each well contains a single-use aliquot of a specific pair of P7 plus P5 primers.

The nucleotide sequence of the index portion of each primer is provided in [Table 51](#) through [Table 58](#). P7 indexes are shown in forward orientation, applicable to any of the supported Illumina platforms. P5 indexes are shown in two orientations (forward and reverse complement) for use with different platforms and sequencing run setup and management tools, e.g., Local Run Manager and Instrument Run Setup. Illumina sequencing platforms and their P5 sequencing orientation are shown in [Table 50](#). Correct representation of the P5 index orientation in sample sheets or during sequencing run setup is crucial to successful demultiplexing. Refer to Illumina support documentation and resources to determine the correct P5 index orientation for your application.

**Table 50** P5 index sequencing orientation by Illumina platform

P5 Index Orientation	Platform
Forward	NovaSeq 6000 with v1.0 chemistry MiSeq HiSeq 2500
Reverse Complement*	NovaSeq 6000 with v1.5 chemistry NextSeq 500/550/1000/2000 HiSeq 3000/4000 iSeq 100 MiniSeq HiSeq X

\* Some run setup and management tools used with these platforms automatically create the reverse complement sequence for the P5 index sequence entered for the run. Be sure to consult Illumina's support documentation for the combination of platform and tools used in your pipeline to determine the correct index orientation to enter during run setup.

## 6 Reference

### SureSelect XT HS2 Index Primer Pair Information

**Table 51 SureSelect XT HS2 Index Primer Pairs 1–48, provided in orange 96-well plate or in strip tubes**

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
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	GCAGGTTT	AGAAGCAA	TTGCTTCT	31	G04	GCAACAAT	TCAGAGGT	ACCTCTGA
8	H01	AAGTGTCT	GCAGGTTT	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	TATTCTTG	CGCTTCCA	TGGAAGCG
22	F03	GCACCTAA	ATCCTCTT	AAGAGGAT	46	F06	CAAGTTAC	TATTCTTG	CAGGAATA
23	G03	TGCTGCTC	GCACCTAA	TTAGGTGC	47	G06	CAGAGCAG	CAAGTTAC	GTAACCTG
24	H03	TGGCACCA	TGCTGCTC	GAGCAGCA	48	H06	CGCGCAAT	CAGAGCAG	CTGCTCTG

Table 52 SureSelect XT HS2 Index Primer Pairs 49–96, provided in orange 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
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	AGTTTCTT
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	GCGTACT	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

## 6 Reference

### SureSelect XT HS2 Index Primer Pair Information

**Table 53 SureSelect XT HS2 Index Primer Pairs 97–144, provided in blue 96-well plate**

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
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	GTTGTCTGA	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	ATCTGCCA	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	ACAGGCCA	143	G06	CTTCACGT	GAATCGCA	TGCGATTC
120	H03	AGACGCCT	GCGCAATT	AATTGCGC	144	H06	CTCCGGTT	CTTCACGT	ACGTGAAG

**Table 54 SureSelect XT HS2 Index Primer Pairs 145–192, provided in blue 96-well plate**

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
145	A07	TGTGACTA	CTCCGGTT	AACCGGAG	169	A10	CGCTCAGA	CTAACAAAG	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	GTA CTCA
152	H07	CACATTCG	TGCAATGC	GCATTGCA	176	H10	TGATTGGC	CGAATCAT	ATGATTCT
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	TGTTCCGG
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	GAGTGCCT	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	TATGTCTT
168	H09	CTAACAAAG	AGGAAGAT	ATCTTCCT	192	H12	GAAGCCTC	GACATCCT	AGGATGTC

## 6 Reference

### SureSelect XT HS2 Index Primer Pair Information

**Table 55 SureSelect XT HS2 Index Primer Pairs 193–240, provided in green 96-well plate**

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
193	A01	GTCTCTTC	GAAGCCTC	GAGGCTTC	217	A04	GCGGTATG	CACGAGCT	AGCTCGTG
194	B01	AGTCACTT	GTCTCTTC	GAAGAGAC	218	B04	TCTATGCG	GCGGTATG	CATACCGC
195	C01	AGCATACA	AGTCACTT	AAGTGA CT	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	AAGGTA CT	AGGCATGC	GCATGCCT
204	D02	AAGCACAA	CATCAGTC	GACTGATG	228	D05	CACTAAGT	AAGGTA CT	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	GTTCTTAA
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	GAGCATA C	GTACCTTG	CAAGGTAC
216	H03	CACGAGCT	ATCTACCA	TGGTAGAT	240	H06	TGCTTACG	GAGCATA C	GTATGCTC

**Table 56 SureSelect XT HS2 Index Primer Pairs 241–288, provided in green 96-well plate**

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
241	A07	AAGAGACA	TGCTTAGC	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	GTTGAGC	GCGAATTA	TAATTCGC
245	E07	CGATTGAA	GTCACAGA	TCTGTGAC	269	E10	GCCAGTAG	GTTGAGC	GCTCGAAC
246	F07	GAGAGATT	CGATTGAA	TTCAATCG	270	F10	AAGGTCGA	GCCAGTAG	CTACTGGC
247	G07	TCATACCG	GAGAGATT	AATCTCTC	271	G10	AGTGAAGT	CACTTATG	CATAAGTG
248	H07	TCCGAAC	TCATACCG	CGGTATGA	272	H10	GTTGCAAG	ATAACGGC	GCCGTTAT
249	A08	AGAGAGAA	TCCGAAC	AGTTCGGA	273	A11	AGCCGAA	GTTGCAAG	CTTGCAAC
250	B08	GATCGTTA	AGAGAGAA	TTCTCTCT	274	B11	AACAGCCG	AGCCGAA	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

## 6 Reference

### SureSelect XT HS2 Index Primer Pair Information

**Table 57 SureSelect XT HS2 Index Primer Pairs 289–336, provided in red 96-well plate**

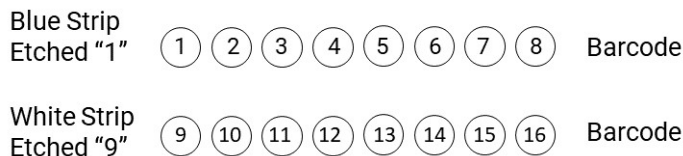
Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
289	A01	AGATAGTG	GATGAGAT	ATCTCATC	313	A04	AGCTACAT	GATCCATG	CATGGATC
290	B01	AGAGGTTA	AGATAGTG	CACTATCT	314	B04	CGCTGTAA	AGCTACAT	ATGTAGCT
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	TCCTCAA	TTGGATCT	AGATCCAA
301	E02	CTCGACAT	CGATACCT	AGGTATCG	325	E05	CGAGTCGA	TCCTCAA	TTGGAGGA
302	F02	GAGATCGC	CTCGACAT	ATGTGCGAG	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 58 SureSelect XT HS2 Index Primer Pairs 337–384, provided in red 96-well plate**

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
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	GTTCAATC	GTCGGTAA	TTACCGAC	367	G10	GCATGCCT	CGTAACTG	CAGTTACG
344	H07	AAGCAGTT	GTTCAATC	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	CAACAAC	GCTATAAG	CTTATAGC
358	F09	CTGAATAG	TCCTGAGA	TCTCAGGA	382	F12	AGAGAATC	CTCTCACT	AGTGAGAG
359	G09	CAAGACTA	CTGAATAG	CTATTCAG	383	G12	TAATGGTC	AGACGAGC	GCTCGTCT
360	H09	GCACAGTA	CAAGACTA	TAGTCTTG	384	H12	GTTGTATC	TAATGGTC	GACCATTA

## Index Primer Pair Strip Tube and Plate Maps

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



**Figure 8** Map of the SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) strip tubes provided with 16 reaction kits

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

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

When using the strip tube-supplied index primer pairs in the library preparation protocol, pierce the foil seal of the appropriate well with a pipette tip just before pipetting the solution. If the foil seal for any unused wells is disrupted during use, the unused wells may be re-sealed using the provided fresh foil seal strips. The provided foil strips may also be used to re-seal used wells to prevent index pair cross-contamination during subsequent use.

See [Table 59](#) on page 91 through [Table 62](#) on page 92 for plate maps showing positions of the SureSelect XT HS2 Index Primer Pairs provided with 96 reaction kits.

### CAUTION

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

**Table 59** 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 60** 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

## 6 Reference

### Index Primer Pair Strip Tube and Plate Maps

**Table 61** 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 62** 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 51](#), 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:
  - Ensure that SureSelect Wash Buffer 2 is pre-warmed to 70°C before use (see [page 54](#)). Select a thermal cycler with a block configured for efficient heating of 0.2 ml liquid volumes; ensure that the plasticware containing the wash buffer is fully seated in the thermal cycler block wells, with minimal liquid volume visible above the block during the pre-warming step.
  - Samples are maintained at 70°C during washes (see [page 55](#))
  - Bead suspensions are mixed thoroughly during washes by pipetting up and down **and** vortexing (see [page 55](#))
- ✓ 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 8](#) to [step 9](#) on [page 52](#)).

### **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 28](#) on [page 49](#) (including segment with one-hour incubation at 65°C), repeat target enrichment using the hybridization program in [Table 29](#) on [page 49](#) (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 29](#) on [page 49](#)).
- ✓ 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
<b>RNA Fragmentation and cDNA Preparation</b>	
Prepare and qualify RNA samples	Prepare 10–200 ng total RNA in 10 µl nuclease-free water (high-quality or FFPE samples). For FFPE RNA, qualify integrity as directed on <a href="#">page 22</a> .
Fragment RNA and prime cDNA synthesis	Add 10 µl 2× Priming Buffer to each sample. Place FFPE samples on ice (further fragmentation not required). <b>For high-quality samples only</b> , fragment by incubating in thermal cycler: 4 min @ 94°C, 1 min @ 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 <sub>2</sub> O, removing 50 µl to fresh well Keep on ice
<b>Library Prep</b>	
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 <sub>2</sub> O, 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 <a href="#">page 40</a>
Purify amplified DNA	50 µl amplified DNA + 50 µl AMPure XP bead suspension Elute DNA in 15 µl nuclease-free H <sub>2</sub> O
Quantify and qualify DNA	Analyze quantity and quality using TapeStation, Bioanalyzer, or Fragment Analyzer System
<b>Hybridization/Capture</b>	
Program thermal cycler	Input the appropriate thermal cycler program on <a href="#">page 49</a> and pause program
Prep DNA in hyb plate	Adjust 200 ng purified prepared library to 12 µl volume with nuclease-free H <sub>2</sub> O
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: <b>Probes ≥3 Mb:</b> 2 µl 25% RNase Block + 5 µl Probe+ 6 µl SureSelect Fast Hybridization Buffer <b>Probes &lt;3 Mb:</b> 2 µl 25% RNase Block + 2 µl Probe + 3 µl nuclease-free H <sub>2</sub> O + 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 <sub>2</sub> O

## 6 Reference

### Quick Reference Protocol

Step	Summary of Conditions
<b>Post-capture amplification</b>	
Prepare PCR master mix	Per reaction: 13 $\mu$ l nuclease-free H <sub>2</sub> O+ 10 $\mu$ l 5 $\times$ Herculase II Reaction Buffer with dNTPs + 1 $\mu$ l SureSelect Post-Capture Primer Mix + 1 $\mu$ l Herculase II Fusion DNA Polymerase Keep on ice
Amplify the bead-bound captured libraries	25 $\mu$ l DNA bead suspension+ 25 $\mu$ l PCR master mix Amplify in thermal cycler using conditions on <a href="#">page 59</a>
Purify amplified DNA	Remove streptavidin beads using magnetic stand; retain supernatant 50 $\mu$ l amplified DNA + 50 $\mu$ l AMPure XP bead suspension Elute DNA in 25 $\mu$ l 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 target-enriched NGS libraries for the Illumina platform.

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