



# SureSelect XT HS2 DNA Kits

## Library Preparation (+/-MBC) / Fast-Hyb Target Enrichment / Pre-capture Pooling Workflow

For Illumina Platform NGS

## Protocol

**Version F0, May 2023**

SureSelect platform manufactured with Agilent SurePrint technology.

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## Edition

Version F0, May 2023

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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 SureSelect XT HS2 DNA Reagent Kits. The specific workflow supported by this guide includes Library Preparation (+/- dual molecular barcodes or MBCs) with Fast-Hyb Target Enrichment and Post-capture library pooling.

### 1 Before You Begin

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

### 2 Input DNA Preparation and Fragmentation

This section describes the steps to prepare and fragment gDNA samples, using either mechanical shearing or enzymatic fragmentation, prior to library preparation.

### 3 Library Preparation using +/- MBC Adaptors

This section describes the steps to prepare dual-indexed gDNA sequencing libraries using either MBC-tagged or MBC-free adaptors.

### 4 Hybridization, Capture, and Amplification

This section describes the steps to hybridize and capture targeted fragments in the prepared DNA library using a SureSelect Probe, using fast (2-3 hour) hybridization conditions and standard SureSelect XT HS2 reagents. Post-capture library amplification through final library QC steps are also included in this section.

### 5 NGS and Analysis Guidelines

This section provides guidelines for post-capture library pooling and downstream NGS sample preparation through analysis.

### 6 Appendix: Using FFPE-derived DNA Samples

This section describes the protocol modifications for gDNA isolated from FFPE samples.

### 7 Reference

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

## What's New in Version F0

- Support for new SureSelect MBC-Free Library Preparation Kits. See [Table 2](#) on page 10 for ordering information. See [Table 52](#) on page 57 and [Table 54](#) on page 58 for kit contents. See [page 23](#) through [page 26](#) for library preparation instructions. See [page 47](#) through [page 51](#) for related NGS support.
- Updates to [Figure 1](#) and [Table 1](#) on page 8
- New table summarizing SureSelect XT HS2 DNA Reagent Kit format options ([Table 2](#) on page 10)
- Support for SureSelect XT HS PreCap Clinical Research Exome V4 ([Table 3](#) on page 11 and [Table 31](#) on page 35)
- Updates to probes supported using this workflow (see [Table 3](#) on page 11). For SureSelect XT PreCap Probes (not listed in [Table 3](#)), see publication [G9957-90000](#) for the recommended overnight hybridization workflow.
- Updates to [Table 5](#) on page 12 to specify reagent amounts suited to the supported run sizes
- Updates to tube cap strip recommendations from domed caps to flat/domed caps based on specific cyclers recommendations ([Table 6](#) on page 12 and [Table 9](#) on page 14)
- New *Troubleshooting* information for users without vacuum concentrator ([Table 6](#) on page 12 and see [page 72](#))
- New Note on [page 15](#) on sample co-processing considerations
- Updates to Covaris instrument setup instructions on [page 18](#)
- Formatting and wording updates to “[Library Preparation using +/- MBC Adaptors](#)” chapter on [page 22](#) to [page 30](#). None of the updates are associated with material changes to the protocol for preparation of SureSelect XT HS2 libraries containing molecular barcodes, or “MBC-tagged” libraries.
- Support for use of new SureSelect MBC-Free Library Preparation Kits for preparation of MBC-Free RNA libraries after dilution of the SureSelect MBC-Free Adaptor Oligo Mix (see [page 23](#) for workflow summary)
- Formatting and wording updates to “[Hybridization, Capture, and Amplification](#)” chapter on [page 33](#) to [page 42](#), including repositioning of post-capture PCR and QC sections into this chapter. None of the updates are associated with material changes to the SureSelect XT HS2 DNA target enrichment or post-capture amplification protocols.
- Updates to downstream sequencing support information (see [page 47](#) to [page 52](#))
- Updates to “[Reagent Kit Contents](#)” on page 57 to [page 58](#)
- Updates to *Troubleshooting*, including considerations for maximizing recovery from AMPure XP purification steps on [page 71](#) and [page 73](#)
- Updates to *Quick Reference Protocol* on [page 75](#) and [page 76](#) including provision of reagent mixture volumes for 96-sample Library Preparation and 12-sample Hybridization runs
- Updates to document cover and format, including new “[NGS and Analysis Guidelines](#)” chapter, replacing the *Post-Capture Sample Processing for Multiplexed Sequencing* chapter in earlier editions
- Updates to [Notices](#) section

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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

This protocol differs from the Illumina Multiplexed Paired-End sequencing manual and other SureSelect protocols at several steps. Pay close attention to the specific adaptor oligo mix used for library preparation and the specific blocking and hybridization reagents used for target enrichment.

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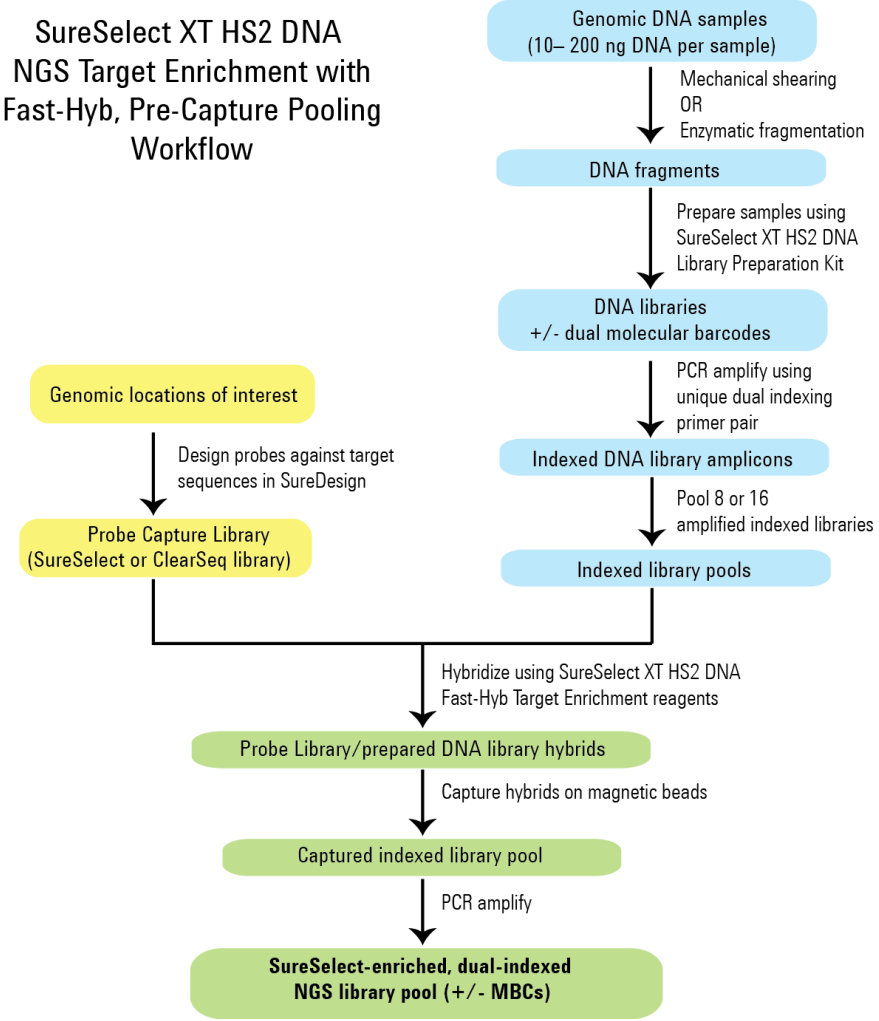
### NOTE

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

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# Overview of the Workflow

The SureSelect XT HS2 DNA workflow is summarized in Figure 1. The estimated time requirements for each step are summarized in Table 1.



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

**Table 1** Estimated time requirements\* (up to 96 sample run size)

Step	Time
DNA fragmentation, library preparation and clean-up	2.5 hours
Pre-capture amplification and clean-up, QC using TapeStation	2 hours
Pooling, Hybridization and Capture	3–4 hours (optional 16-hour hold available for 2-day workflow)
Post-capture amplification and clean-up, QC using TapeStation	1.5 hours

\* Estimates are provided as guidelines for 96 Library Prep/12 Hyb reaction runs using 200 ng high-quality input DNA, enzymatic DNA fragmentation, and probe design >5 Mb. Your results may vary.



## Procedural and Safety 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 nuclease 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.
- Several reagent solutions used in the SureSelect XT HS2 protocols are highly viscous. Make sure to follow the mixing instructions provided in the protocols.
- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of 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.

### CAUTION

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

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## SureSelect XT HS2 DNA Kits and Supported Workflows

SureSelect XT HS2 DNA kits are available in a variety of kit configurations supporting several workflow options. This publication provides optimized protocols for workflows that include SureSelect XT HS2 DNA library preparation (+/- duplex molecular barcodes or MBCs) with Fast-Hyb Target Enrichment and Pre-capture library pooling.

To determine the materials needed for your research design, first select the appropriate SureSelect XT HS2 DNA Kits for the Fast-Hyb/Pre-capture Pooling workflow from [Table 2](#). Make sure that your selections include both Library Preparation and Target Enrichment reagents. See [Table 3](#) for a list of SureSelect XT HS PreCap Probes recommended for use in this workflow. If your research design includes target enrichment of SureSelect XT HS2 libraries with SureSelect XT PreCap Probes not listed in [Table 3](#), see publication [G9957-90000](#) for the recommended workflow.

**Table 2** Ordering Information for SureSelect XT HS2 DNA Kits for Fast-Hyb, Pre-capture Pooling Workflow

Agilent Part Number (Index Pairs Supplied)	Samples Processed	Library Prep Kit (Adaptor Type)	Target Enrichment Kit
<b>SureSelect XT HS2 DNA Library Preparation Kits for MBC-tagged Libraries (Target Enrichment Kit purchased separately)</b>			
G9985A (Index 1–96)	96*	✓	✗
G9985B (Index 97–192)		(MBC-tagged)	(order G9987A, below)
G9985C (Index 193–288)			
G9985D (Index 289–384)			
<b>SureSelect XT HS2 DNA Library Preparation Kits for MBC-free Libraries (Target Enrichment Kit purchased separately)</b>			
G9956A (Index 1–96)	96*	✓	✗
G9956B (Index 97–192)		(MBC-Free)	(order G9987A, below)
G9956C (Index 193–288)			
G9956D (Index 289–384)			
<b>SureSelect XT HS2 Fast-Hyb Target Enrichment Kit (Library Preparation Kit purchased separately)</b>			
G9987A	12 Hybs <sup>†</sup>	✗	✓
		(order one of G9985A–D <b>OR</b> G9956A–D, above)	(Fast-Hyb, Pre-Cap Pool)

\* The minimum supported Library Prep run size for 96-reaction kits is 24 samples per run, with kits containing enough reagents for 4 runs of 24 samples each. Reagent use volumes for this option are provided in the library preparation protocol section, along with reagent use volumes for the single 96-sample run configuration that is typically optimal for the pre-capture pooling workflow. The prepared libraries are pooled in groups of 8 or 16 for hybridization, depending on the hybridization probe design (see [Table 4](#) on page 11).

† 12-Hyb Target Enrichment Kits support target enrichment for 96 samples pre-capture pooled using the configuration of either 12 pools containing 8 samples/pool or 6 pools containing 16 samples/pool based on the probe design(s) used in the run (see [Table 4](#) on page 11).

**Table 3** Compatible Probes

Probe Capture Library	Design ID	Part Number/Ordering Information
<b>Pre-designed Probes</b>		
SureSelect XT HS PreCap Human All Exon V8 (12 Hybs)*	S33266340	5191-6877
SureSelect XT HS PreCap Human All Exon V8+UTR (12 Hybs)*	S33613271	5191-7405
SureSelect XT HS PreCap Human All Exon V8+NCV (12 Hybs)*	S33699751	5191-7411
SureSelect XT HS PreCap Clinical Research Exome V4 (12 Hybs)*	S34226467	5280-0024
SSel XT HS PreCap Human All Exon V7 (12 Hybs)*	S31285117	5191-5735
<b>Custom Probes</b>		
SSEL PreCap Custom Tier1 1–499 kb (6 Hybs or 30 Hybs)*		
SSEL PreCap Custom Tier2 0.5 –2.9 Mb (6 Hybs or 30 Hybs)*		Please visit the <a href="#">SureDesign website</a> to design Custom SureSelect XT HS probes and obtain ordering information. Contact the SureSelect support team (see <a href="#">page 2</a> ) or your local representative if you need assistance.
SSEL PreCap Custom Tier3 3 –5.9 Mb (6 Hybs or 30 Hybs)*		
SSEL PreCap Custom Tier4 6 –11.9 Mb (6 Hybs or 30 Hybs)*		
SSEL PreCap Custom Tier5 12–24 Mb (6 Hybs or 30 Hybs)*		

\* See [Table 4](#) for samples processed and supported run configuration details for each probe pack size. For example, the 12-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 8 samples/pool (12 hybs × 8 samples/hyb). Probe vials contain enough reagents for the run configurations listed in [Table 4](#) using the run setup on [page 37](#).

**Table 4** Supported Run Configurations for Probes used in the Pre-capture Pooling Workflow

Probe Type	Pack Size	Samples Pooled per Hybridization	Total Samples Processed	Supported Run Sizes
SureSelect XT HS PreCap All Exon and Exome Probes	12 Hybs	8	96	2 runs x 6 hybs/run 1 run x 12 hybs/run
SureSelect XT HS PreCap Custom Probes	6 Hybs	16	96	1 run x 6 hybs/run
	30 Hybs	16	480	5 runs x 6 hybs/run

## Additional Materials Required

The additional materials required to complete the SureSelect XT HS2 protocol will vary based on the following considerations:

- DNA sample type: high-quality gDNA derived from fresh/fresh-frozen samples vs. FFPE-derived gDNA samples
- DNA fragmentation method used in workflow: mechanical (Covaris-mediated) shearing vs. enzymatic fragmentation

Refer to [Table 5](#) through [Table 8](#) for additional materials needed to complete the protocols using the selected DNA sample type/fragmentation method.

**Table 5** Required Reagents--All Sample Types/Fragmentation Methods

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	—
Qubit BR dsDNA Assay Kit, 100 assays	Thermo Fisher Scientific p/n Q32850	—
Nuclease-free Water	Thermo Fisher Scientific p/n AM9930	Water should not be DEPC-treated
AMPure XP Kit, 60 mL	Beckman Coulter Genomics p/n A63881	—
Dynabeads MyOne Streptavidin T1, 2 mL	Thermo Fisher Scientific p/n 65601	—

**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 (see [Table 6](#)).

**Table 6** Required Equipment--All Sample Types/Fragmentation Methods

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 (flat or domed)*	Consult the thermal cycler manufacturer's recommendations
Nucleic acid analysis system (instrument and consumables)	Select one system from <a href="#">Table 8</a> on page 13
Qubit Fluorometer	Thermo Fisher Scientific p/n Q33238
Qubit Assay Tubes	Thermo Fisher Scientific p/n Q32856
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
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
Magnetic separator	Thermo Fisher Scientific p/n 12331D or equivalent <sup>†</sup>
Vacuum concentrator <sup>‡</sup>	Savant SpeedVac, model DNA120, or equivalent
Multichannel and single channel pipettes	Rainin Pipet-Lite Multi Pipette or equivalent
Sterile, nuclease-free aerosol barrier pipette tips, vortex mixer, ice bucket, and powder-free gloves	General laboratory supplier

\* Consult the thermal cycler manufacturer's recommendations for use of either flat or domed strip caps and for any accessories (e.g., compression mats) required for optimal performance with the selected caps. Ensure that the combination of selected thermal cycler and plasticware provides complete sealing of sample wells and optimal contact between the instrument heated lid and vial cap for heat transfer.

† 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.

‡ Prepared library pools may require dehydration prior hybridization, which uses 1.5 or 3  $\mu$ g of library pool in a 12  $\mu$ L volume. If no vacuum concentrator is available in your laboratory, see [Troubleshooting](#) on [page 72](#) for suggested protocol modifications.

**Table 7** Additional Required Materials based on Sample Type/Fragmentation Method

Description	Vendor and Part Number	Usage Notes
<b>Required for preparation of high-quality DNA samples (not required for FFPE DNA sample preparation)</b>		
High-quality gDNA purification system, for example: QIAamp DNA Mini Kit, 50 Samples	Qiagen, p/n 51304	—
<b>Required for preparation of FFPE DNA samples (not required for high-quality DNA sample preparation)</b>		
QIAamp DNA FFPE Tissue Kit, 50 Samples	Qiagen p/n 56404	Recommended reagents for FFPE gDNA sample preparation prior to library preparation.
Deparaffinization Solution	Qiagen p/n 19093	
FFPE DNA integrity assessment system: Agilent NGS FFPE QC Kit, 96 reactions OR TapeStation Genomic DNA Analysis Consumables: Genomic DNA ScreenTape Genomic DNA Reagents	Agilent p/n G9700A  Agilent p/n 5067-5365 p/n 5067-5366	Recommended systems for FFPE gDNA qualification prior to library preparation.
<b>DNA fragmentation options</b>		
Mechanical DNA fragmentation system: Covaris Sample Preparation System Covaris microTUBE sample holders	Covaris model E220 Covaris p/n 520045	Not required for workflows using enzymatic DNA fragmentation. Additional Covaris instrument models and sample holders may be used after optimization of shearing conditions.
SureSelect Enzymatic Fragmentation Kit, 96 Reactions	Agilent p/n 5191-4080	Not required for workflows using mechanical (Covaris-mediated) DNA shearing.

**Table 8** Nucleic Acid Analysis Platform Options--Select One

Analysis System	Vendor and Part Number Information
Agilent 4200/4150 TapeStation Instrument Consumables: 96-well sample plates 96-well plate foil seals 8-well tube strips 8-well tube strip caps D1000 ScreenTape D1000 Reagents High Sensitivity D1000 ScreenTape High Sensitivity D1000 Reagents	Agilent p/n G2991AA/G2992AA  p/n 5042-8502 p/n 5067-5154 p/n 401428 p/n 401425 p/n 5067-5582 p/n 5067-5583 p/n 5067-5584 p/n 5067-5585
Agilent 2100 Bioanalyzer Instrument Agilent 2100 Expert SW Laptop Bundle (optional) Consumables: DNA 1000 Kit High Sensitivity DNA Kit	p/n G2939BA p/n G2953CA  p/n 5067-1504 p/n 5067-4626
Agilent 5200/5300/5400 Fragment Analyzer Instrument Consumables: NGS Fragment Kit (1-6000 bp) HS NGS Fragment Kit (1-6000 bp)	Agilent p/n M5310AA/M5311AA/M5312AA  p/n DNF-473-0500 p/n DNF-474-0500

## Optional Materials

**Table 9** 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 46</a> )
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

## 2 Input DNA Preparation and Fragmentation

- Step 1. Prepare and qualify the genomic DNA samples 16
  - Preparation of high-quality gDNA from fresh biological samples 16
  - Preparation and qualification of gDNA from FFPE samples 16
- Step 2. Fragment the DNA 18
  - Method 1: Mechanical DNA shearing with Covaris 18
  - Method 2: Enzymatic DNA fragmentation 20

This section describes the steps to prepare, quantify, qualify and fragment input DNA samples prior to SureSelect XT HS2 library preparation and target enrichment. Protocols are provided for two alternative methods of DNA fragmentation—mechanical shearing or enzymatic DNA fragmentation.

The library preparation protocol is compatible with both high-quality gDNA prepared from fresh or fresh-frozen samples and lower-quality DNA prepared from FFPE samples. Modifications required for FFPE samples are included throughout the protocol steps. For a summary of modifications for FFPE samples see [Chapter 6](#), “Appendix: Using FFPE-derived DNA Samples” on [page 53](#).

The library preparation protocol requires 10 ng to 200 ng of input DNA, with adjustments to DNA input amount or quantification method required for some FFPE samples. For optimal sequencing results, use the maximum amount of input DNA available within the recommended range.

### NOTE

Consider whether your data analysis application has any requirements for sample co-processing at this stage. Requirements for Agilent’s Alissa Reporter applications are outlined on [page 50](#).

# Step 1. Prepare and qualify the genomic DNA samples

## Preparation of high-quality gDNA from fresh biological samples

- 1 Prepare high-quality gDNA using a suitable purification system, such as Qiagen's QIAamp DNA Mini Kit, following the manufacturer's protocol.

### NOTE

Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0.

- 2 Use the Qubit BR dsDNA Assay Kit to determine the concentration of each gDNA sample. Follow the manufacturer's instructions for the instrument and assay kit.

Additional qualification of DNA samples is not required for DNA derived from fresh biological samples. Proceed to "Step 2. Fragment the DNA" on page 18.

## Preparation and qualification of gDNA from FFPE samples

- 1 Prepare gDNA from FFPE tissue sections using Qiagen's QIAamp DNA FFPE Tissue Kit and Qiagen's Deparaffinization Solution, following the manufacturer's protocol. Elute the final gDNA samples from the MinElute column in two rounds, using 30  $\mu$ L Buffer ATE in each round, for a final elution volume of approximately 60  $\mu$ L.

### NOTE

If tissue lysis appears incomplete after one hour of digestion with Proteinase K, add an additional 10  $\mu$ L of Proteinase K and continue incubating at 56°C, with periodic mixing, for up to three hours.

Store the gDNA samples on ice for same-day library preparation, or at -20°C for later processing.

- 2 Use the Qubit BR dsDNA Assay Kit to determine the concentration of each gDNA sample. Follow the manufacturer's instructions for the instrument and assay kit.
- 3 Assess the quality (DNA integrity) for each FFPE DNA sample using one of the methods below.

### Option 1: Qualification using the Agilent NGS FFPE QC Kit

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include a  $\Delta\Delta$ Cq DNA integrity score and the precise quantity of amplifiable DNA in the sample, allowing direct normalization of DNA input for each sample.

- a Analyze a 1- $\mu$ L aliquot of each FFPE gDNA sample using the Agilent NGS FFPE QC Kit. Follow the instructions provided in the [kit user manual](#).
- b Use the  $\Delta\Delta$ Cq score-based guidelines below (summarized in [Table 10](#)) to determine the appropriate input DNA quantification method for your sample:

For all samples with  $\Delta\Delta$ Cq DNA integrity score  $\leq 1$  (more intact FFPE DNA samples), use the Qubit-based gDNA concentration to determine volume of input DNA needed for the protocol.

For all samples with  $\Delta\Delta$ Cq DNA integrity score  $> 1$  (less intact FFPE DNA samples), use the qPCR-based concentration of amplifiable gDNA, reported by the Agilent NGS FFPE QC Kit results, to determine amounts of input DNA for the protocol.



**Table 10** DNA input guidelines based on  $\Delta\Delta Cq$  DNA integrity score

$\Delta\Delta Cq$ Score	DNA Input Guidelines
$\Delta\Delta Cq \leq 1^*$	10 ng to 200 ng DNA, based on Qubit Assay quantification
$\Delta\Delta Cq > 1$	10 ng to 200 ng of amplifiable DNA, based on qPCR quantification

\* FFPE samples with  $\Delta\Delta Cq$  scores  $\leq 1$  should be treated like non-FFPE samples for DNA input amount determinations. For samples of this type, make sure to use the DNA concentration determined by the Qubit Assay, instead of the concentration determined by qPCR, to calculate the volume required for 10–200 ng DNA.

**Option 2: Qualification using the Agilent Genomic DNA ScreenTape assay DIN score**

The Agilent TapeStation Genomic DNA ScreenTape assay provides a quantitative electrophoretic assay for DNA sample integrity determination. This assay reports a DNA Integrity Number (DIN) score for each sample which is used to estimate the appropriate normalization of DNA input required for low-integrity DNA samples.

- a Analyze a 1- $\mu$ L aliquot of each FFPE gDNA sample using the Genomic DNA ScreenTape assay. Follow the instructions provided in the [assay Quick Guide](#).
- b Consult [Table 11](#) for DIN score-based input DNA input guidelines.

**Table 11** SureSelect XT HS2 DNA input modifications based on DNA Integrity Number (DIN) score

Protocol Parameter	non-FFPE Samples	FFPE Samples		
		DIN > 8*	DIN 3–8	DIN < 3
DNA input for Library Preparation	10 ng to 200 ng DNA, quantified by Qubit Assay	10 ng to 200 ng DNA, quantified by Qubit Assay	Use at least 15 ng for more intact samples and at least 40 ng for less intact samples. Use the maximum amount of DNA available, up to 200 ng, for all samples. Quantify by Qubit Assay.	Use at least 50 ng for more intact samples and at least 100 ng for the least intact samples. Use the maximum amount of DNA available, up to 200 ng, for all samples. Quantify by Qubit Assay.

\* FFPE samples with  $DIN > 8$  should be treated like non-FFPE samples for DNA input amount determinations.

## Step 2. Fragment the DNA

### Method 1: Mechanical DNA shearing with Covaris

In this step, gDNA samples are sheared using conditions optimized for either high-quality or FFPE DNA in a 50- $\mu$ L shearing volume.

The target fragment size and corresponding shearing conditions may vary for workflows using different NGS read lengths. See [Table 12](#) for a summary of shearing duration recommendations. Complete shearing instructions are also provided below.

**Table 12** Covaris shearing duration based on NGS read length

NGS read length requirement	Target fragment size	Shearing duration for high-quality DNA samples	Shearing duration for FFPE DNA samples*
2 x 100 reads	150 to 200 bp	2 x 120 seconds	240 seconds
2 x 150 reads	180 to 250 bp	2 x 60 seconds	240 seconds

\* For FFPE DNA samples, initial DNA fragment size may impact the post-shear fragment size distribution, resulting in fragment sizes shorter than the target ranges listed in this table. All FFPE samples should be sheared for 240 seconds to generate fragment ends suitable for library construction. Libraries prepared from FFPE samples should be analyzed using an NGS read length suitable for the final library fragment size distribution.

Before you begin, set up the Covaris instrument according to the manufacturer's instructions. Allow enough time (typically 30–60 minutes) for instrument degassing and water bath chilling before starting the protocol.

#### NOTE

This protocol has been optimized using a Covaris model E220 instrument and the 130- $\mu$ l Covaris microTUBE. Consult the manufacturer's recommendations for use of other Covaris instruments or sample holders to achieve the same target DNA fragment size.

- 1 Prepare the DNA samples for the run by diluting 10–200 ng of each gDNA sample with 1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA) to a final volume of 50  $\mu$ L. Vortex well to mix, then spin briefly to collect the liquid. Keep the samples on ice.

#### NOTE

**Do not dilute samples to be sheared using water.** Shearing samples in water reduces the overall library preparation yield and complexity.

- 2 Complete the DNA shearing steps below for each gDNA sample.
  - a Transfer the 50- $\mu$ L DNA sample into a Covaris microTUBE.
  - b Spin the microTUBE for 30 seconds to collect the liquid and to remove any bubbles from the bottom of the tube.

- c Secure the microTUBE in the tube holder and shear the DNA with the settings in [Table 13](#).

**Table 13** Shear settings for Covaris E-series instrument (SonoLab software v7 or later)

Setting	High-quality DNA for 2 × 100 read NGS	High-quality DNA for 2 × 150 read NGS	FFPE DNA (2 × 100 or 2 × 150 read NGS)
Duty Factor	10%	10%	10%
Peak Incident Power (PIP)	175	175	175
Cycles per Burst	200	200	200
Treatment Time	2 × 120 seconds (see two-round instructions below)	2 × 60 seconds (see two-round instructions below)	240 seconds
Bath Temperature	2° to 8° C	2° to 8° C	2° to 8° C

Use the steps below for two-round shearing of high-quality DNA samples only:

- Shear for 120 or 60 seconds (see [Table 13](#))
  - Spin the microTUBE for 10 seconds
  - Vortex the microTUBE at high speed for 5 seconds
  - Spin the microTUBE for 10 seconds
  - Shear for additional 120 or 60 seconds
  - Spin the microTUBE for 10 seconds
  - Vortex the microTUBE at high speed for 5 seconds
  - Spin the microTUBE for 10 seconds
- d** After completing the shearing step(s), put the Covaris microTUBE back into the loading and unloading station.
- e** While keeping the snap-cap on, insert a pipette tip through the pre-split septum, then slowly remove the sheared DNA.
- f** Transfer the sheared DNA sample (approximately 50 µL) to a 96-well plate or strip tube sample well. Keep the samples on ice.
- g** After transferring the DNA sample, spin the microTUBE briefly to collect any residual sample volume. Transfer any additional collected liquid to the sample well used in [step f](#).

**NOTE**

It is important to avoid loss of input DNA at this step, especially for low-abundance DNA samples. Visually inspect the microTUBE to ensure that all of the sample has been transferred. If droplets remain in the microTUBE, repeat [step g](#).

The 50-µL sheared DNA samples are now ready for NGS sequencing library preparation, beginning with end repair/dA-tailing. Proceed to [“Library Preparation using +/- MBC Adaptors”](#) on page 22.

**NOTE**

This is not a stopping point in the workflow, and analysis of the sheared samples is not required before they are used for library preparation. Proceed directly to [page 22](#).

## Method 2: Enzymatic DNA fragmentation

In this step, gDNA samples are fragmented using Agilent's SureSelect Enzymatic Fragmentation Kit.

- 1 In wells of a thermal cycler-compatible strip tube or PCR plate, dilute 10 to 200 ng of each gDNA sample with nuclease-free water or 1X Low TE Buffer to a final volume of 7  $\mu$ L.  
If the DNA concentration is too low to supply 10–200 ng input DNA in 7  $\mu$ L, sample volume may be reduced using a suitable concentration method. Alternatively, see *Troubleshooting* on [page 71](#) for protocol modifications for dilute samples.
- 2 Thaw the vial of 5X SureSelect Fragmentation Buffer, vortex, then place on ice.
- 3 Preprogram a thermal cycler as shown in [Table 14](#); pause until use in [step 7](#). Optimal fragmentation conditions vary based on the NGS read length to be used in the workflow. Refer to [Table 15](#) for the duration at 37°C appropriate for your sample type and required NGS read length.

**Table 14** Thermal cycler program for enzymatic fragmentation (10  $\mu$ L vol)

Step	Temperature	Time
Step 1	37°C	Varies--see <a href="#">Table 15</a>
Step 2	65°C	5 minutes
Step 3	4°C	Hold

**Table 15** Fragmentation duration based on sample type and NGS read length

NGS read length requirement	Target fragment size	Duration of 37°C incubation step ( <a href="#">Table 14</a> )	
		High-quality DNA samples	FFPE DNA samples*
2 $\times$ 100 reads	150 to 200 bp	15 minutes	15 minutes
2 $\times$ 150 reads	180 to 250 bp	10 minutes	15 minutes

\* For FFPE DNA samples, initial DNA fragment size may impact the post-fragmentation size distribution, resulting in fragment sizes shorter than the target ranges listed in this table. All FFPE samples should be incubated at 37°C for 15 minutes to generate fragment ends suitable for library construction. Libraries prepared from FFPE samples should be analyzed using an NGS read length suitable for the final library fragment size distribution.

- 4 Prepare the appropriate volume of Fragmentation master mix by combining the reagents in [Table 16](#). Mix well by pipetting up and down 20 times or seal the tube and vortex at high speed for 5–10 seconds. Spin briefly to remove any bubbles and keep on ice.

**Table 16** Preparation of Fragmentation master mix

Reagent	Volume for 1 reaction	Volume for 8 reactions* (includes excess)	Volume for 24 reactions† (includes excess)
5X SureSelect Fragmentation Buffer	2 $\mu$ L	18 $\mu$ L	50 $\mu$ L
SureSelect Fragmentation Enzyme	1 $\mu$ L	9 $\mu$ L	25 $\mu$ L
Total	3 $\mu$ L	27 $\mu$ L	75 $\mu$ L

\* 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.

- 5 Add 3  $\mu\text{L}$  of the fragmentation master mix to each sample well containing 7  $\mu\text{L}$  of input DNA.
- 6 Mix well by pipetting up and down 20 times or cap the wells and vortex at high speed for 5–10 seconds. Spin the samples briefly.
- 7 Immediately place the samples in the thermal cycler and resume the enzymatic fragmentation program in [Table 14](#).
- 8 When the program reaches the 4°C Hold step, remove the samples from the thermal cycler, add 40  $\mu\text{L}$  of nuclease-free water to each sample, and place the samples on ice.

The 50- $\mu\text{L}$  reactions are now ready for NGS sequencing library preparation, beginning with end repair/dA-tailing. Proceed to “[Library Preparation using +/- MBC Adaptors](#)” on page 22.

**NOTE**

This is not a stopping point in the workflow, and analysis of the enzymatically-fragmented samples is not required before they are used for library preparation. Proceed directly to [page 22](#).

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### 3

## Library Preparation using +/- MBC Adaptors

- Step 1. Prepare the ligation master mix 24
- Step 2. Repair and dA-tail the DNA 3' ends 24
- Step 3. Ligate the adaptor 25
- Step 4. Purify libraries using AMPure XP Beads 26
- Step 5. Amplify and index the pre-capture libraries 27
- Step 6. Purify amplified libraries using AMPure XP Beads 29
- Step 7. QC and quantify the pre-capture libraries 30

In this workflow segment, NGS libraries are prepared from the gDNA fragments. Libraries can be prepared using adaptors that either include or exclude molecular barcodes (MBCs). After adaptor ligation, the libraries are amplified using dual indexing primers. For each sample to be sequenced, an individual dual-indexed library is prepared.

To process multiple samples, the protocol includes steps for preparation of reagent mixtures with overage, which are afterward distributed to the DNA library samples. Mixtures for preparation of 24 or 96 samples are shown in tables as examples.

The NGS library preparation protocol requires fragmented DNA samples produced by mechanical shearing ([page 18](#) to [page 19](#)) or by enzymatic fragmentation ([page 20](#) to [page 21](#)). Samples produced by either method should contain 10–200 ng of DNA fragments in a volume of 50 µL.

The MBC-free library preparation kit supported by this publication may also be used to prepare MBC-free RNA sequencing libraries from cDNA fragments prepared according to the [SureSelect XT HS2 RNA](#) protocol. For cDNA library preparation, the SureSelect MBC-Free Adaptor Oligo Mix must be diluted before use as described on [page 23](#).

This workflow segment uses the components listed in [Table 17](#). Remove the listed reagents from cold storage, and prepare as directed before use (refer to the *Where Used* column).

**Table 17** Reagents thawed before use in protocol

Storage Location	Kit Component	Preparative Steps	Where Used
<p><b>For MBC-tagged libraries</b>, get reagents from the <i>SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR)</i> box, stored at <math>-20^{\circ}\text{C}</math></p> <p><b>For MBC-free libraries</b>, get reagents from the <i>SureSelect XT HS2 Library Preparation Kit for ILM, MBC-Free (Pre PCR)</i> box, stored at <math>-20^{\circ}\text{C}</math></p>	Ligation Buffer (bottle)	Thaw on ice (may require $>20$ minutes) then keep on ice, vortex to mix	<a href="#">page 24</a>
	T4 DNA Ligase (blue cap)	Place on ice just before use, invert to mix	<a href="#">page 24</a>
	End Repair-A Tailing Buffer (bottle)	Thaw on ice (may require $>20$ minutes) then keep on ice, vortex to mix	<a href="#">page 25</a>
	End Repair-A Tailing Enzyme Mix (orange cap)	Place on ice just before use, invert to mix	<a href="#">page 25</a>
	<p><b>For MBC-tagged libraries:</b> XT HS2 Adaptor Oligo Mix (white cap)</p> <p><b>For MBC-free libraries:</b> SureSelect MBC-Free Adaptor Oligo Mix (black cap)</p>	Thaw on ice then keep on ice, vortex to mix	<a href="#">page 26</a>
	Herculase II Fusion DNA Polymerase (red cap)	Place on ice just before use, mix by pipetting	<a href="#">page 28</a>
	5x Herculase II Buffer with dNTPs (clear cap)	Thaw on ice then keep on ice, vortex to mix	<a href="#">page 28</a>
$-20^{\circ}\text{C}$	SureSelect XT HS2 Index Primer Pairs for ILM (select the specific set of indexes to be used in the run): Index Pairs 1-96 (orange plate) Index Pairs 97-192 (blue plate) Index Pairs 193-288 (green plate) Index Pairs 289-384 (red plate)	Thaw on ice then keep on ice, vortex to mix	<a href="#">page 29</a>
$+4^{\circ}\text{C}$	AMPure XP Beads	Equilibrate at room temperature (RT) for at least 30 minutes before use, vortex to mix	<a href="#">page 26</a> and <a href="#">page 29</a>

**Additional preparative step for MBC-free RNA library construction:** To prepare MBC-free RNA libraries from cDNA, dilute the provided SureSelect MBC-Free Adaptor Oligo Mix 5-fold with 1X Low TE Buffer before use.

For a 96 reaction run, combine 100  $\mu\text{L}$  of SureSelect MBC-Free Adaptor Oligo Mix and 400  $\mu\text{L}$  of 1X Low TE Buffer. Vortex to mix and keep on ice until use in [step 3](#) on [page 26](#). Prepare a fresh dilution of the SureSelect MBC-Free Adaptor Oligo Mix for each run.

After completing this adaptor dilution step, MBC-free RNA libraries may be prepared according to the standard [SureSelect XT HS2 RNA Reagent Kits protocol](#) using the diluted SureSelect MBC-Free Adaptor Oligo Mix instead of the XT HS2 RNA Adaptor Oligo Mix (green-cap).

## Step 1. Prepare the ligation master mix

Prepare the ligation master mix to allow equilibration to room temperature while you are completing the end repair/dA-tailing step. Leave DNA samples on ice while completing this step.

### CAUTION

The Ligation Buffer used in this step is viscous. Make sure to follow the mixing instructions in [step 1](#) and [step 2](#) below.

- 1 Vortex the thawed vial of Ligation Buffer for 15 seconds at high speed just before use.
- 2 Prepare the appropriate volume of ligation master mix by combining the reagents in [Table 18](#).

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.

**Keep at room temperature for 30–45 minutes before use on [page 25](#).**

**Table 18** Preparation of ligation master mix

Reagent	Volume for 1 reaction	Volume for 24 reactions* (includes excess)	Volume for 96 reactions (includes excess)
Ligation Buffer (bottle)	23 µL	598 µL	2300 µL
T4 DNA Ligase (blue cap)	2 µL	52 µL	200 µL
Total	25 µL	650 µL	2500 µL

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

## Step 2. Repair and dA-tail the DNA 3' ends

### CAUTION

The End Repair-A Tailing Buffer used in this step is viscous. Make sure to follow the mixing instructions in [step 2](#) and [step 3](#) on [page 25](#).

- 1 Preprogram a thermal cycler as shown in [Table 19](#); pause until use in [step 5](#).

**Table 19** Thermal cycler program for end repair/dA-tailing (70 µl vol)

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



- 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.
- Prepare the appropriate volume of dA-tailing master mix by combining the reagents in [Table 20](#).

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 with a pipette set to at least 80% of the mixture volume, or seal the tube and vortex at high speed for 5–10 seconds. Spin briefly and keep on ice.

**Table 20** Preparation of end repair/dA-tailing master mix

Reagent	Volume for 1 reaction	Volume for 24 reactions (includes excess)	Volume for 96 reactions (includes excess)
End Repair-A Tailing Buffer (bottle)	16 $\mu$ L	416 $\mu$ L	1600 $\mu$ L
End Repair-A Tailing Enzyme Mix (orange cap)	4 $\mu$ L	104 $\mu$ L	400 $\mu$ L
Total	20 $\mu$ L	520 $\mu$ L	2000 $\mu$ L

- Add 20  $\mu$ L of the end repair/dA-tailing master mix to each sample well containing 50  $\mu$ L of DNA fragments. 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.
- Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in [Table 19](#).

### Step 3. Ligate the adaptor

- Once the thermal cycling program in [Table 19](#) reaches the 4°C Hold step, transfer the samples to ice. Preprogram the cycler as show in [Table 21](#); pause until use in [step 4](#).

**Table 21** Thermal cycler program for ligation (100  $\mu$ l vol)

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

- To each end-repaired/dA-tailed DNA sample (approximately 70  $\mu$ L), add 25  $\mu$ L of the ligation master mix that was prepared on [page 24](#) and kept at room temperature. Mix by pipetting up and down at least 10 times using a pipette set to 70  $\mu$ L or cap the wells and vortex at high speed for 5–10 seconds. Briefly spin the samples.

- 3 Add 5  $\mu$ L of the appropriate SureSelect Adaptor Oligo Mix to each sample:
  - For **MBC-tagged** libraries—5  $\mu$ L of XT HS2 Adaptor Oligo Mix (white-capped tube)
  - For **MBC-free** libraries—5  $\mu$ L of SureSelect MBC-Free Adaptor Oligo Mix (black-capped tube)

Mix by pipetting up and down 15–20 times using a pipette set to 70  $\mu$ L or cap the wells and vortex at high speed for 5–10 seconds.

**NOTE**

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

For MBC-free RNA library preparation from cDNA samples, make sure to dilute the MBC-Free Adaptor Oligo Mix 5-fold (see [page 23](#)) before use and follow the [SureSelect XT HS2 RNA Reagent Kits protocol](#) for all subsequent steps.

- 4 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in [Table 21](#).

**NOTE**

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

## Step 4. Purify libraries using AMPure XP Beads

Once the thermal cycler program in [Table 21](#) reaches the 4°C hold step, purify the libraries using room-temperature AmpPure XP Beads.

Critical purification protocol parameters are summarized for experienced users in [Table 22](#).

**Table 22** 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 Prepare 400  $\mu$ L of 70% ethanol per sample, plus excess, for use in [step 8](#).

**NOTE**

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

- 2 Mix the room-temperature AMPure XP Beads well until homogeneous and consistent in color.
- 3 Transfer the DNA samples from the thermal cycler to room temperature, then add 80  $\mu$ L of the bead suspension to each sample well.
- 4 Mix by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds then spin briefly to collect the samples, being careful not to pellet the beads.
- 5 Incubate the bead suspensions 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 fresh 70% ethanol in each sample well.
- 9 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 10 Repeat [step 8](#) and [step 9](#) once for a total of two washes.
- 11 Cap the wells, then briefly spin the samples to collect the residual ethanol. Return the samples to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

**NOTE**

In the drying step below, do not dry the bead pellet to the point that the pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

- 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 (up to 2 minutes).  
  
Samples can instead be dried by keeping the unsealed plate or strip tube on the benchtop for approximately 5 minutes or until the residual ethanol has just evaporated.
- 13 Elute the library DNA by adding 35  $\mu$ L of nuclease-free water to each sample well.
- 14 Mix by pipetting up and down 10–15 times or cap the wells and vortex at high speed 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. If samples were vortexed, spin briefly to collect the liquid, being careful not to pellet the beads.
- 15 Incubate for 2 minutes at room temperature.
- 16 Put the plate or strip tube in the magnetic stand and leave until the solution is clear (up to 5 minutes).
- 17 Remove the cleared supernatant (approximately 34  $\mu$ L) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

## Step 5. Amplify and index the pre-capture libraries

- 1 Determine the appropriate index pair assignment for each sample. See [Table 59](#) on page 61 through [Table 66](#) on page 68 for nucleotide sequences of the 8 bp index portion of the primers used to amplify the DNA libraries in this step.

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

**NOTE**

Agilent's SureSelect XT HS2 index pairs use a uniform numbering system across all platforms and formats. For example, index pairs 1-8 provided in orange plates in 96-reaction kits are equivalent to index pairs 1-8 provided in blue strip tubes in 16-reaction kits and to index pairs 1-8 provided in Magnis automation system black index strips (labeled *D1*). Do not combine samples indexed with the same-numbered index pair from different kit formats for multiplex sequencing.

**CAUTION**

The SureSelect XT HS2 Index Primer Pairs are provided in single-use aliquots. To avoid cross-contamination of libraries, do not retain and re-use any residual volume for subsequent experiments.

2 Preprogram a thermal cycler as shown in [Table 23](#); pause until use in [step 6](#).

**Table 23** Pre-capture PCR thermal cycler program (50 µl vol; heated lid ON)

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8 to 14, based on input DNA quality and quantity (see <a href="#">Table 24</a> )	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

**Table 24** Pre-capture PCR cycle number recommendations

Quality of Input DNA	Quantity of Input DNA	Cycles
Intact DNA from fresh sample	100 to 200 ng	8 cycles
	50 ng	9 cycles
	10 ng	11 cycles
FFPE sample DNA	100 to 200 ng*	11 cycles
	50 ng*	12 cycles
	10 ng*	14 cycles

\* qPCR-determined quantity of amplifiable DNA or DIN value-adjusted amount of input DNA

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

**Table 25** Preparation of pre-capture PCR reaction mix

Reagent	Volume for 1 reaction	Volume for 24 reactions (includes excess)	Volume for 96 reactions (includes excess)
5x Herculase II Buffer with dNTPs (clear cap)	10 µL	260 µL	1000 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	26 µL	100 µL
<b>Total</b>	<b>11 µL</b>	<b>286 µL</b>	<b>1100 µL</b>

4 Add 11 µL of the PCR reaction mixture prepared in [Table 25](#) to each sample well containing purified DNA library (34 µL).

- 5 Add 5  $\mu$ L 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 23](#) 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.

**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.

**NOTE**

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

## Step 6. Purify amplified libraries using AMPure XP Beads

Once the thermal cycler program in [Table 23](#) reaches the 4°C hold step, purify the libraries using room-temperature AmpPure XP Beads.

Critical purification protocol parameters are summarized for experienced users in [Table 26](#).

**Table 26** 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 Prepare 400  $\mu$ L of 70% ethanol per sample, plus excess, for use in [step 8](#).
- 2 Mix the room-temperature AMPure XP Beads well until homogeneous and consistent in color.
- 3 Transfer the library DNA samples from the thermal cycler to room temperature, then add 50  $\mu$ L of the bead suspension to each sample well.
- 4 Mix by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds then spin briefly to collect the samples, being careful not to pellet the beads.
- 5 Incubate the bead suspensions 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 fresh 70% ethanol in each sample well.
- 9 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 10 Repeat [step 8](#) and [step 9](#) once for a total of two washes.

- Cap the wells, then briefly spin the samples to collect the residual ethanol. Return the samples to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

**NOTE**

In the drying step below, do not dry the bead pellet to the point that the pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

- 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).

Samples can instead be dried by keeping the unsealed plate or strip tube on the benchtop for approximately 5 minutes or until the residual ethanol has just evaporated.

- Elute the library DNA by adding 15 µL of nuclease-free water to each sample well.
- Mix by pipetting up and down 10–15 times or cap the wells and vortex at high speed 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. If samples were vortexed, spin briefly to collect the liquid, being careful not to pellet the beads.
- Incubate for 2 minutes at room temperature.
- Put the plate or strip tube in the magnetic stand and leave until the solution is clear (up to 5 minutes).
- Remove the cleared supernatant (approximately 15 µL) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

**Stopping Point** If you do not plan to continue through the hybridization step on same day, seal the wells and store at 4°C overnight or at –20°C for prolonged storage (remove aliquot for QC analysis before storage, if appropriate).

## Step 7. QC and quantify the pre-capture libraries

Analyze a sample of each library using one of the platforms listed in [Table 27](#). Follow the instructions in the linked user guide provided for each assay.

**Table 27** Pre-capture library analysis options

Analysis platform	Assay used at this step	Link to assay instructions	Amount of library sample to analyze
Agilent 4200/4150 TapeStation system	D1000 ScreenTape	<a href="#">Agilent D1000 Assay Quick Guide</a>	1 µL of five-fold dilution
Agilent 2100 Bioanalyzer system	DNA 1000 Kit	<a href="#">Agilent DNA 1000 Kit Guide</a>	1 µL of five-fold dilution
Agilent 5200/5300/5400 Fragment Analyzer system	NGS Fragment Kit (1-6000 bp)	<a href="#">Agilent NGS Fragment Kit (1-6000 bp) Kit Guide</a>	2 µL of five-fold dilution

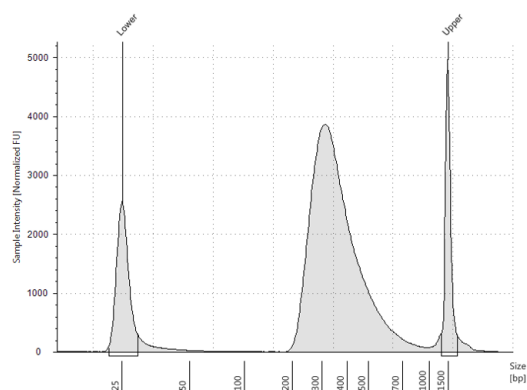
Each analysis method provides an electropherogram showing the size distribution of fragments in the sample and tools for determining the concentration of DNA in the sample. See [Table 28](#) for fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided in [Figure 2](#) through [Figure 4](#) to illustrate typical results.

Determine the concentration of the library DNA by integrating under the entire peak.

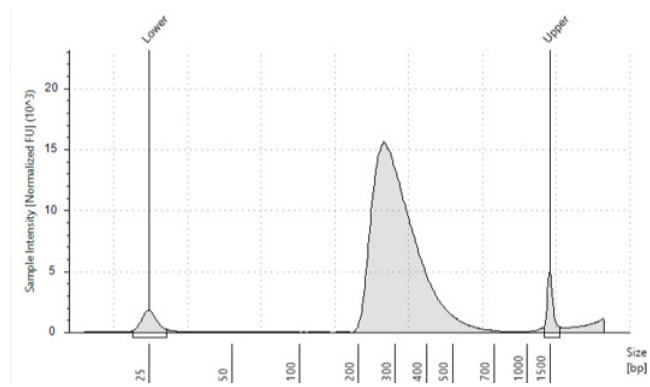
**Table 28** Pre-capture library qualification guidelines

NGS read length used for fragmentation protocol selection	Fragmentation method	Input DNA type	Expected average fragment size for pre-capture library
2 ×100 reads	Mechanical shearing	Intact DNA	300 to 375 bp
		FFPE DNA	300 to 450 bp
	Enzymatic fragmentation	Intact DNA	325 to 400 bp
		FFPE DNA	300 to 450 bp
2 ×150 reads	Mechanical shearing	Intact DNA	350 to 450 bp
		FFPE DNA	300 to 450 bp
	Enzymatic fragmentation	Intact DNA	350 to 450 bp
		FFPE DNA	300 to 450 bp

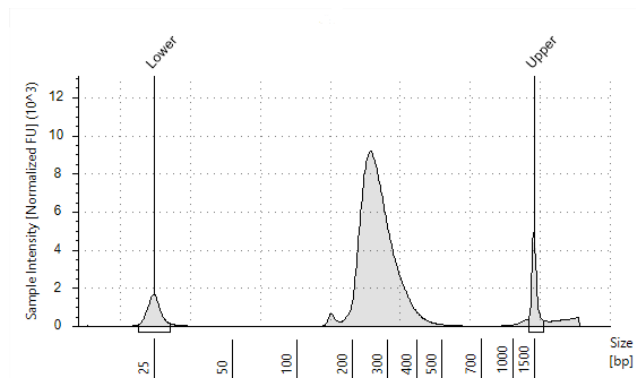
Observation of a low molecular weight peak, in addition to the expected library fragment peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to that seen in example electropherogram in [Figure 4](#). See *Troubleshooting* on [page 72](#) for additional considerations.



**Figure 2** Pre-capture library prepared from a sheared high-quality gDNA sample analyzed using a D1000 ScreenTape assay.



**Figure 3** Pre-capture library prepared from a typical FFPE gDNA sample (fragmented by shearing) analyzed using a D1000 ScreenTape assay.



**Figure 4** Pre-capture library prepared from a low-quality FFPE gDNA sample (fragmented by shearing) analyzed using a 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.



## 4 Hybridization, Capture, and Amplification

- Step 1. Pool indexed DNA samples for hybridization 34
- Step 2. Hybridize library pools to the SureSelect Probe 35
- Step 3. Prepare streptavidin beads for capture 38
- Step 4. Capture the hybridized library pools 38
- Step 5. Amplify the captured library pools 39
- Step 6. Purify the final library pools using AMPure XP Beads 41
- Step 7. QC and quantify final libraries 42

In this workflow segment, the prepared indexed libraries are pooled in sets of 8 or 16 indexes and then pools are hybridized with a target-specific probe. After hybridization, the targeted molecules are captured on streptavidin-coated beads and then PCR-amplified.

The recommended number of indexes combined per pool varies for different probes. See [Table 30](#) for pooling recommendations.

The standard single-day SureSelect XT HS2 Fast-Hyb workflow includes the hybridization step immediately followed by capture and amplification. If required, after hybridization under the Fast-Hyb conditions, 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 36](#).

The hybridization and capture workflow segments use the components listed in [Table 29](#). Remove the listed reagents from cold storage, where required, and prepare as directed before use (refer to the *Where Used* column).

**Table 29** Reagents for Hybridization and Capture

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), stored at -20°C	SureSelect XT HS2 Blocker Mix (blue cap)	Thaw and keep on ice, vortex to mix	<a href="#">page 36</a>
	SureSelect RNase Block (purple cap)	Thaw and keep on ice, vortex to mix	<a href="#">page 36</a>
	SureSelect Fast Hybridization Buffer (bottle)	Thaw and keep at room temperature	<a href="#">page 37</a>
-80°C	SureSelect Probe	Thaw and keep on ice, vortex to mix	<a href="#">page 37</a>
+4°C	Dynabeads MyOne Streptavidin T1 Beads	Remove from 4°C just before use, vortex to mix	<a href="#">page 38</a>
SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), stored at RT	SureSelect Binding Buffer (bottle)	Ready to use	<a href="#">page 38</a>
	SureSelect Wash Buffer 1 (bottle)	Ready to use	<a href="#">page 39</a>
	SureSelect Wash Buffer 2 (bottle)	Ready to use	<a href="#">page 38</a>

## Step 1. Pool indexed DNA samples for hybridization

In this segment of the workflow, you pool the indexed library samples, then adjust the pool volume to 12 µL before hybridization to the probe.

Each hybridization reaction requires a total of either 3 µg or 1.5 µg pooled indexed libraries, made up of equal amounts of 8 or 16 individual libraries, based on the probe design. See [Table 30](#) for probe-specific pool composition recommendations.

**Table 30** Pre-capture pooling recommendations

Probe description	Total amount of indexed library pool used for hybridization	Number of indexed libraries per pool	Amount of each library in pool
SureSelect XT HS PreCap Human All Exon V8/V8+UTR/V8+NCV or SureSelect Clinical Research Exome V4	3 µg	8	375 ng
SureSelect XT HS PreCap Human All Exon V7	1.5 µg	8	187.5 ng
SureSelect XT HS PreCap Custom Probes	1.5 µg	16	93.75 ng

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

Each final pool should contain either 3 µg or 1.5 µg of indexed DNA (see [Table 30](#)).

### NOTE

It is acceptable to include greater amounts of DNA in the library pools if desired. Make sure to combine all libraries in the pool in equivalent amounts.

- Bring the volume of each pool to 12  $\mu$ L with nuclease-free water.

For pool volumes >12  $\mu$ L, use a vacuum concentrator held at  $\leq 45^\circ\text{C}$  to dehydrate the prepped library pools to volume <12  $\mu$ L without completely drying the samples. Measure the final volume in each well and then adjust each volume to 12  $\mu$ L with nuclease-free water.

**NOTE**

If a vacuum concentrator is not available in your laboratory, see *Troubleshooting* on page 72.

- Cap the wells, then vortex the strip tube or plate vigorously for 30 seconds. Spin in a centrifuge or mini-plate spinner to collect the liquid, then keep the samples on ice until use in [step 2](#) on page 36.

## Step 2. Hybridize library pools to the SureSelect Probe

- Preprogram a thermal cycler (with heated lid ON) with the program in [Table 31](#) for the SureSelect XT HS Human All Exon V8 Probes (V8/V8+UTR/V8+NCV) and SureSelect XT HS Clinical Research Exome V4, or with the program in [Table 32](#) for all other probes. Pause the cycler until samples are loaded in [step 3](#).

**Table 31** Hybridization program for **SureSelect XT HS Human All Exon V8/V8+UTR/V8+NCV or Clinical Research Exome V4 Probes** (30  $\mu$ l vol; heated lid ON)

Segment Number	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	65°C	10 minutes
3	1	65°C	Hold for reagent addition (see <a href="#">step 6</a> on <a href="#">page 37</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 38</a>

**Table 32** Hybridization program for **all other SureSelect XT HS probes\*** (30  $\mu$ l vol; heated lid ON)

Segment Number	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	65°C	10 minutes
3	1	65°C	Hold for reagent addition (see <a href="#">step 6</a> on <a href="#">page 37</a> )
4	60	65°C	1 minute
		37°C	3 seconds
5	1	65°C <sup>†</sup>	Hold briefly until ready to begin capture steps on <a href="#">page 38</a>

\* The program used in this table is optimal for SureSelect XT HS probe designs with a Fast Hybridization workflow. SureSelect XT probe designs typically have improved performance using the Overnight Hybridization workflow described in G9957-90000.

**NOTE**

The protocol may be modified for a 2-day workflow with an overnight pause by making the following changes:

- In the final segment of the thermal cycler program (Table 31 or Table 32), replace the final brief 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 38 on day 2, after the overnight hold.

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

**CAUTION**

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

- 3 Transfer the sealed sample plate or strip to the thermal cycler and resume the thermal cycling program in Table 31 or Table 32, allowing the cycler to complete Segments 1 and 2 of the program and then initiate the Segment 3 Hold.

**Important: Additional reagents are added to sample wells in the thermal cycler during the Segment 3 Hold step.** Begin preparing the additional hybridization reagents during Segments 1 and 2 as described in step 4 and step 5 below. If needed, you can finish these preparation steps while the thermal cycler holds samples at 65°C in Segment 3.

- 4 Prepare a 25% solution of SureSelect RNase Block (1 part RNase Block to 3 parts water) according to Table 33. Prepare the amount required for the number of hybridization reactions in the run, plus excess. Mix well and keep on ice.

**Table 33** Preparation of RNase Block solution

Reagent	Volume for 1 reaction	Volume for 6 Hyb reactions (includes excess)	Volume for 12 Hyb reactions (includes excess)
SureSelect RNase Block	0.5 µL	3.5 µL	7.0 µL
Nuclease-free water	1.5 µL	10.5 µL	21.0 µL
Total	2 µL	14 µL	28 µL

**NOTE**

Prepare the mixture described in [step 5](#), below, just before addition to DNA samples in [step 6](#) during the thermal cycler program Segment 3 Hold. Keep the mixture at room temperature only briefly during preparative steps. Do not keep solutions containing the probe at room temperature for extended periods.

- 5 Prepare the Probe Hybridization Mix appropriate for your probe design size. Use [Table 34](#) for probes  $\geq 3$  Mb or [Table 35](#) for probes  $< 3$  Mb. For custom probes, see the probe tube label for design size range.

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

**Table 34** Preparation of Probe Hybridization Mix for probes  $\geq 3$  Mb

Reagent	Volume for 1 reaction	Volume for 6 Hyb reactions (includes excess)	Volume for 12 Hyb reactions (includes excess)
25% RNase Block solution (from <a href="#">step 4</a> )	2 $\mu$ L	14 $\mu$ L	26 $\mu$ L
Probe (with design $\geq 3$ Mb)	5 $\mu$ L	35 $\mu$ L	65 $\mu$ L
SureSelect Fast Hybridization Buffer	6 $\mu$ L	42 $\mu$ L	78 $\mu$ L
<b>Total</b>	<b>13 <math>\mu</math>L</b>	<b>91 <math>\mu</math>L</b>	<b>169 <math>\mu</math>L</b>

**Table 35** Preparation of Probe Hybridization Mix for probes  $< 3$  Mb

Reagent	Volume for 1 reaction	Volume for 6 Hyb reactions (includes excess)	Volume for 12 Hyb reactions (includes excess)
25% RNase Block solution (from <a href="#">step 4</a> )	2 $\mu$ L	14 $\mu$ L	26 $\mu$ L
Probe (with design $< 3$ Mb)	2 $\mu$ L	14 $\mu$ L	26 $\mu$ L
SureSelect Fast Hybridization Buffer	6 $\mu$ L	42 $\mu$ L	78 $\mu$ L
Nuclease-free water	3 $\mu$ L	21 $\mu$ L	39 $\mu$ L
<b>Total</b>	<b>13 <math>\mu</math>L</b>	<b>91 <math>\mu</math>L</b>	<b>169 <math>\mu</math>L</b>

- 6 After the thermal cycler starts the Segment 3 Hold, while keeping the DNA + Blocker samples in the cycler, transfer 13  $\mu$ L of the room-temperature probe hybridization mix from [step 5](#) to each sample well.

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

The hybridization reaction wells now contain approximately 30  $\mu$ L.

- 7 Seal the wells with fresh strip caps. Make sure that all wells are completely sealed. Vortex briefly, then spin the plate or strip tube briefly to remove any bubbles from the bottom of the wells. Immediately return the plate or strip tube to the thermal cycler.
- 8 Resume the thermal cycling program to allow hybridization of the prepared library 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  $\mu$ L is lost to evaporation under the conditions used for hybridization.

### Step 3. Prepare streptavidin beads for capture

#### NOTE

If performing same-day hybridization and capture, begin the bead preparation steps below approximately one hour after starting hybridization in [step 8](#) above. 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 below.

- 1 Vigorously resuspend the vial of streptavidin beads or on a vortex mixer. The magnetic beads settle during storage.
- 2 For each hybridization sample, add 50  $\mu\text{L}$  of the resuspended beads to wells of a fresh PCR plate or strip tube.
- 3 Wash the beads:
  - a Add 200  $\mu\text{L}$  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  $\mu\text{L}$  of SureSelect Binding Buffer.

#### NOTE

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

### Step 4. Capture the hybridized library pools

- 1 After all streptavidin bead preparation steps are complete, and with the hybridization thermal cycling program in the final hold segment (see [Table 31](#) or [Table 32](#) on page 35), transfer the samples to room temperature.
- 2 Immediately transfer the entire volume (approximately 30  $\mu\text{L}$ ) of each hybridization mixture to wells containing 200  $\mu\text{L}$  of washed streptavidin beads using a multichannel pipette.

Pipette up and down 5–8 times to mix then seal the wells with fresh 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- $\mu\text{L}$  aliquots of Wash Buffer 2 in wells of a fresh 96-well plate or strip tubes. Aliquot 6 wells of buffer for each DNA 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 (approximately 1 to 2 minutes), then remove and discard 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 the supernatant.

### CAUTION

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

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

Do not use a tissue incubator, 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 steps below.
  - a Resuspend the beads in 200  $\mu$ L of 70°C prewarmed Wash Buffer 2. Pipette up and down 15–20 times, until beads are fully resuspended.
  - b 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 on 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  $\mu$ L of nuclease-free water to each sample well. Pipette up and down 8 times to resuspend the beads.
- 11 Keep the samples on ice until they are used in the PCR reactions below.

### NOTE

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

## Step 5. Amplify the captured library pools

This workflow segment uses the components listed in [Table 36](#). Remove the listed reagents from cold storage and prepare as directed before use (refer to the *Where Used* column).

**Table 36** Reagents for post-capture amplification

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), stored at –20°C	Herculase II Fusion DNA Polymerase (red cap)	Place on ice just before use, pipette to mix	<a href="#">page 40</a>
	5× Herculase II Buffer with dNTPs (clear cap)	Thaw and keep on ice, vortex to mix	<a href="#">page 40</a>
	SureSelect Post-Capture Primer Mix (clear cap)	Thaw and keep on ice, vortex to mix	<a href="#">page 40</a>
+4°C	AMPure XP Beads	Equilibrate at room temperature for at least 30 minutes before use, vortex to mix	<a href="#">page 41</a>

- 1 Preprogram a thermal cycler as shown in [Table 37](#); pause until use in [step 5](#).

**Table 37** Post-Capture PCR thermal cycler program (50 µl vol; heated lid ON)

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10–16 (see <a href="#">Table 38</a> for probe design size-based cycle number recommendation)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

**Table 38** Post-capture PCR cycle number recommendations

Probe Design Size	Cycles
Probes <0.2 Mb	16 cycles
Probes 0.2–3 Mb	12–16 cycles
Probes 3–5 Mb	11–12 cycles
Probes >5 Mb (including Human All Exon and Exome probes)	10–11 cycles

- 2 Prepare the appropriate volume of post-capture PCR reaction mix, as described in [Table 39](#), on ice. Mix well on a vortex mixer.

**Table 39** Preparation of post-capture PCR reaction mix

Reagent	Volume for 1 reaction	Volume for 6 reactions (includes excess)	Volume for 12 reactions (includes excess)
Nuclease-free water	13 µL	91 µL	182 µL
5× Herculase II Buffer with dNTPs (clear cap)	10 µL	70 µL	140 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	7 µL	14 µL
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	7 µL	14 µL
<b>Total</b>	<b>25 µL</b>	<b>175 µL</b>	<b>350 µL</b>

- 3 Add 25 µL of the PCR reaction mix prepared in [Table 39](#) to each sample well containing 25 µL of bead-bound target-enriched DNA.
- 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 37](#).



- 6 When the PCR amplification program is complete, spin the plate or strip tube briefly. Remove the streptavidin beads by placing the plate or strip tube on the magnetic stand at room temperature. Wait 2 minutes for the solution to clear.
- 7 **Transfer each supernatant (approximately 50 µL) to wells of a fresh plate or strip tube.** The streptavidin beads can be discarded at this time.

**CAUTION**

Make sure to retain the supernatant for each sample at this step for further processing.

**NOTE**

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

**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 6. Purify the final library pools using AMPure XP Beads

Purify the amplified libraries using room-temperature AmpPure XP Beads. Critical purification protocol parameters are summarized for experienced users in [Table 40](#).

**Table 40** AMPure XP bead cleanup parameters after post-capture PCR

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

- 1 Prepare 400 µL of 70% ethanol per sample, plus excess, for use in [step 8](#).
- 2 Mix the room-temperature AMPure XP Beads well until homogeneous and consistent in color.
- 3 Add 50 µL of the bead suspension to each amplified DNA sample (approximately 50 µL) in the PCR plate or strip tube well.
- 4 Mix by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds then spin briefly to collect the samples, being careful not to pellet the beads.
- 5 Incubate the bead suspensions 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 2 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 touch the beads while removing the solution.
- 8 Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 µL of fresh 70% ethanol in each sample well.
- 9 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 10 Repeat [step 8](#) and [step 9](#) once for a total of two washes.

- 11 Cap the wells, then briefly spin the samples to collect the residual ethanol. Return the samples to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

**NOTE**

In the drying step below, do not dry the bead pellet to the point that the pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

- 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 (up to 2 minutes).

Samples can instead be dried by keeping the unsealed plate or strip tube on the benchtop for approximately 5 minutes or until the residual ethanol has just evaporated.

- 13 Elute the library DNA by adding 25 µL of Low TE buffer to each sample well.
- 14 Mix by pipetting up and down 10–15 times or cap the wells and vortex at high speed 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. If samples were vortexed, spin briefly to collect the liquid, being careful not to pellet the beads.
- 15 Incubate for 2 minutes at room temperature.
- 16 Put the plate or strip tube in the magnetic stand and leave until the solution is clear (up to 5 minutes).
- 17 Remove the cleared supernatant (approximately 25 µL) 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 plan to continue through the library pooling for NGS step on same day, seal the wells and store at 4°C overnight or at –20°C for prolonged storage (remove aliquot for QC analysis before storage, if appropriate).

## Step 7. QC and quantify final libraries

Analyze a sample of each library using one of the platforms listed in [Table 41](#). Follow the instructions in the linked user guide provided for each assay.

**Table 41** Post-capture library analysis options

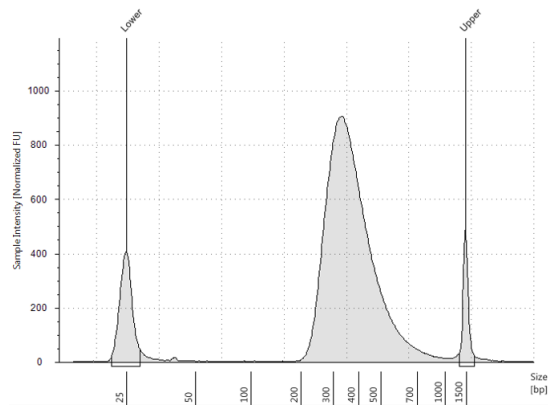
Analysis platform	Assay used at this step	Link to assay instructions	Amount of library sample to analyze
Agilent 4200/4150 TapeStation system	High Sensitivity D1000 ScreenTape	<a href="#">Agilent High Sensitivity D1000 Assay Quick Guide</a>	2 µL
Agilent 2100 Bioanalyzer system	High Sensitivity DNA Kit	<a href="#">Agilent High Sensitivity DNA Kit Guide</a>	1 µL
Agilent 5200/5300/5400 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

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 42](#) for fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided in [Figure 5](#) through [Figure 7](#) to illustrate typical results.

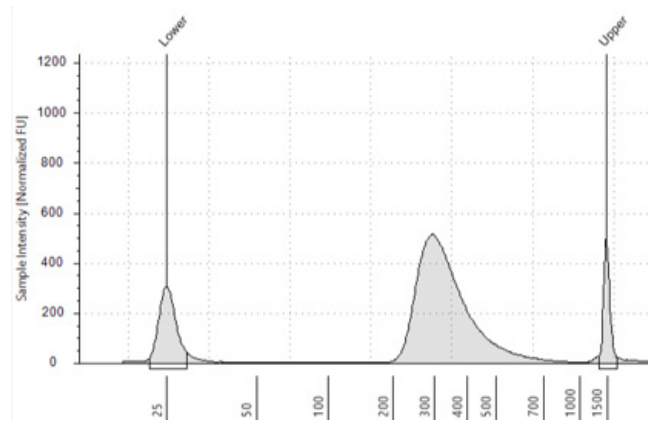
Determine the concentration of the library DNA by integrating under the entire peak.

**Table 42** Post-capture library qualification guidelines

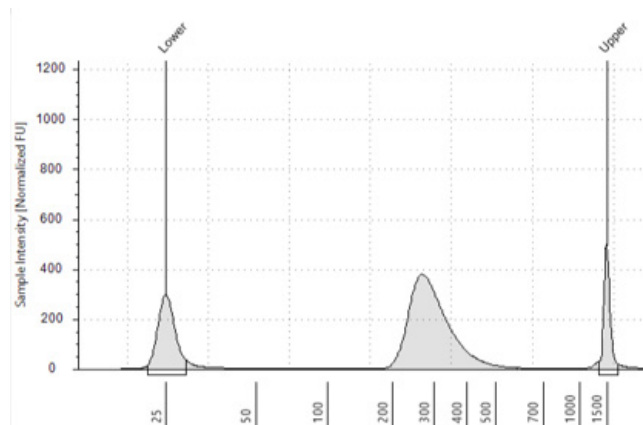
NGS read length used for fragmentation protocol selection	Input DNA type	Expected DNA fragment size peak position
2 × 100 reads	Intact DNA	300 to 400 bp (see <a href="#">Figure 5</a> for sample electropherogram)
	FFPE DNA	200 to 400 bp (see <a href="#">Figure 6</a> and <a href="#">Figure 7</a> for sample electropherograms)
2 × 150 reads	Intact DNA	330 to 450 bp
	FFPE DNA	200 to 450 bp



**Figure 5** Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.



**Figure 6** Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.



**Figure 7** Post-capture library prepared from a low-quality FFPE gDNA 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.

## 5 NGS and Analysis Guidelines

Step 1. Optional: Pool samples for multiplexed sequencing 46

Step 2. Prepare the sequencing samples 47

Step 3. Sequence the libraries 48

Step 4. Process and analyze the reads 49

This section provides guidelines for the NGS and analysis segments of the workflow.

## Step 1. Optional: Pool samples for multiplexed sequencing

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

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

If samples will not be further combined in post-capture pools, proceed to [page 47](#).

Combine the libraries such that each indexed library is present in equimolar amounts in the pool using one of the following methods. Use the diluent specified by your sequencing provider, such as Low TE, for the dilution steps.

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

**Method 2:** Starting with library 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 43](#) shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20  $\mu\text{L}$  at 10 nM DNA.

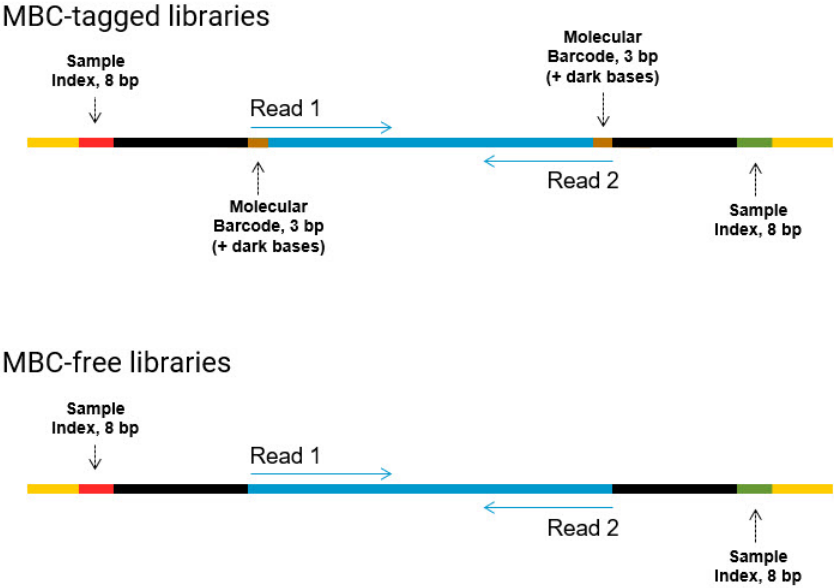
**Table 43** Example of volume calculation for total volume of 20  $\mu\text{L}$  at 10 nM concentration

Component	V(f)	C(i)	C(f)	#	Volume to use ( $\mu\text{L}$ )
Sample 1	20 $\mu\text{L}$	20 nM	10 nM	4	2.5
Sample 2	20 $\mu\text{L}$	10 nM	10 nM	4	5
Sample 3	20 $\mu\text{L}$	17 nM	10 nM	4	2.9
Sample 4	20 $\mu\text{L}$	25 nM	10 nM	4	2
Low TE					7.6

If you store the library pool before sequencing, add Tween 20 to 0.1% v/v and store at  $-20^{\circ}\text{C}$  short term, or store under the conditions specified by your sequencing provider.

# Step 2. Prepare the sequencing samples

The final SureSelect XT HS2 library pool is ready for 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 Illumina sequencers, as shown in Figure 8.



**Figure 8** Content of SureSelect XT HS2 sequencing libraries. Each fragment contains one target insert (blue) surrounded by the following elements: Illumina paired-end sequencing elements (black), unique dual indexes (red and green), library PCR primers (yellow), and optional molecular barcodes (brown).

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. [Table 44](#) provides guidelines for use of several instrument and chemistry combinations suitable for this application. For other Illumina NGS platforms, consult Illumina’s documentation for kit configuration and seeding concentration guidelines.

**Table 44** Illumina Kit Configuration Selection Guidelines

Instrument	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
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
iSeq 100	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	50–150 pM
NextSeq 500/550	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2.5	1.2–1.5 pM
NextSeq 1000/2000	All Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1, v2, or v3	650–1000 pM
HiSeq 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	200 or 300 Cycle Kit	v1.0 or v1.5	300–600 pM
NovaSeq 6000	Xp Workflow Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1.0 or v1.5	200–400 pM
NovaSeq X	All runs	2 × 100 bp or 2 × 150 bp	100, 200 or 300 Cycle Kit	v1	90–180 pM

Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in [Table 44](#) or provided by Illumina. Follow Illumina’s recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

### Step 3. Sequence the libraries

Set up the sequencing run to generate Read 1 and Read 2 FASTQ files for each sample using the instrument’s software in standalone mode or using an Illumina run management tool such as Local Run Manager (LRM), Illumina Experiment Manager (IEM) or BaseSpace. Enter the appropriate **Cycles** or **Read Length** value for your library read length and using 8-bp dual index reads. See [Table 45](#) showing example settings for 2x150 bp sequencing.

**Table 45** Run settings for 2x150 bp sequencing

Run Segment	Cycles/Read Length
Read 1	151*
Index 1 (i7)	8
Index 2 (i5)	8
Read 2	151*

\* Follow Illumina’s recommendation to add one (1) extra cycle to the desired read length.



Follow Illumina's instructions for each platform and setup software option, incorporating the additional setup guidelines below:

- Each of the sample-level indexes (i7 and i5) requires an 8-bp index read. For complete index sequence information, see [Table 59](#) on page 61 through [Table 66](#) on page 68.
- No custom primers are used for SureSelect XT HS2 library sequencing. Leave all *Custom Primers* options for *Read 1*, *Read 2*, *Index 1* and *Index 2* primers cleared/deselected during run setup.
- For MBC-tagged libraries, turn off any adaptor trimming tools included in Illumina's run setup and read processing software applications. Adaptors are trimmed in later processing steps using Agilent software tools to ensure proper processing of the adaptors, including the degenerate molecular barcodes (MBCs) in the adaptor sequences.
- For runs set up using Illumina's LRM, IEM, or BaseSpace applications, refer to Illumina's instructions and support resources for setting up runs with custom library prep kits and index kits in the selected software. For use in these applications, the SureSelect XT HS2 index sequences provided in [Table 59](#) through [Table 66](#) should be converted to .tsv/.csv file format or copied to a Sample Sheet according to Illumina's specifications for each application. If you need assistance with SureSelect XT HS2 run setup in your selected application (e.g., generating index files or Sample Sheet templates), contact the SureSelect support team (see [page 2](#)) or your local representative.

## Step 4. Process and analyze the reads

Guidelines are provided below for typical NGS read processing and analysis pipeline steps appropriate for SureSelect XT HS2 DNA libraries. Your NGS analysis pipeline may vary.

- 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. For MBC-tagged libraries, turn off the MBC/UMI trimming options in Illumina's demultiplexing software to allow proper adaptor processing and use of the MBCs by Agilent's NGS software tools.
- The demultiplexed FASTQ data needs to be pre-processed to remove sequencing adaptors and extract the MBC sequences, if present. Both of the Agilent NGS software tools described below include the correct read pre-processing steps for SureSelect XT HS2 DNA libraries.
- Agilent's Alissa Reporter software provides a variety of applications for the complete FASTQ file to variant discovery process for SureSelect-enriched libraries. See [page 50](#) for more information.
- Agilent's Genomics NextGen Toolkit (AGeNT) software modules provide the tools to process the library read FASTQ files to analysis-ready BAM files for germline or somatic variant analysis workflows. See [page 51](#) for more information.
- If you have prepared MBC-tagged libraries, but your sequence analysis pipeline excludes MBCs and is incompatible with Alissa Reporter and AGeNT software, you can trim or mask the first five bases from each read before alignment as described in the *Note* on [page 52](#).

## Using Agilent's Alissa Reporter software for SureSelect XT HS2 DNA NGS workflows

Alissa Reporter software provides a complete FASTQ-to-Report solution for Agilent's SureSelect assays, processing NGS data from FASTQ format to VCF format, and reporting human germline SNV, InDel and CNV calls.

### NOTE

Analysis for detection of somatic variants in FFPE-derived or other DNA samples is not supported by Alissa Reporter at the time of this publication. Please visit the [Alissa Reporter page at www.agilent.com](http://www.agilent.com) for the most up-to-date information on supported applications and workflows, including somatic variant analysis applications, expected in summer 2023.

Alissa Reporter is a cloud-based, multi-tenant software as a service (SaaS) product, delivering integrated pre-processing of SureSelect XT HS2 DNA library reads (adaptor trimming, MBC extraction and de-duplication) along with secondary data analysis and quality control (QC) analytics using a built-in dashboard. To obtain more information and to purchase access to the software please visit the [Alissa Reporter page at www.agilent.com](http://www.agilent.com).

Key considerations for SureSelect XT HS2 DNA assay steps prior to Alissa Reporter software analysis are summarized below:

- Determine the sample co-processing requirements for your application. Sample requirements for germline application CNV analysis are outlined below. Somatic applications (expected in summer 2023) directed to tumor/normal analysis require co-processing of the tumor sample and a matched/unmatched reference (normal) sample in the same SureSelect NGS library preparation run. Consult the Alissa Reporter software Help topics for the current software version for detailed information on sample requirements for the available applications.
- Alissa Reporter makes germline CNV calls using a co-analysis strategy in which unrelated samples from the same run are used to determine the reference signal for the target sample (no specific reference sample is required for the germline applications). At least 3 and preferably 8 or more unrelated samples need to be analyzed in Alissa Reporter together to obtain a reliable reference signal for CNV calls. For CNV calling on the X and Y chromosomes, unrelated samples of the same gender are required. For best results, process the samples to be used for CNV co-analysis in the same SureSelect run and in the same sequencing run in order to minimize any processing-based variance.
- Alissa Reporter v1.1 and later includes applications for germline analysis of human DNA libraries enriched using a pre-designed or custom SureSelect human probe. Libraries enriched using SureSelect XT HS Human All Exon V7 or V8 probes are analyzed with the corresponding *Human All Exon V7 Germline* or *Human All Exon V8 Germline* application in Alissa Reporter. Libraries enriched using other probes, including additional pre-designed probes, are analyzed using an Alissa Reporter *Custom* application. The Alissa Reporter console provides tools for importing both pre-designed and custom probe designs from SureDesign and setting up a new *Custom* application for each imported design.
- When setting up FASTQ file uploads for each sample, select the **Application Chemistry** menu option required for your adaptor type, as shown in [Table 46](#).

**Table 46** Alissa Reporter Application Chemistry selection based on Adaptor type

HS2 Library Adaptor Type	Application Chemistry Selection Required
MBC-free	<i>XTHS2 (no MBC)</i> *
MBC-tagged	<i>XTHS2</i>

\* Available in Alissa Reporter software v1.2 and later.

- Unmerged and merged FASTQ files are supported. Upload of BAM files or other non-FASTQ file formats is not supported.
- Obtain any required sequence file parameters (e.g., file size or read number limits) from the Alissa Reporter software Help topics or the [Alissa Reporter Release Notes](#) for the current software version. Key FASTQ file parameters for Alissa Reporter version 1.1 are provided in [Table 47](#).

**Table 47** FASTQ file parameters for Alissa Reporter software v1.1

Parameter	Value(s)	Notes
Maximum file size	50 GB/file	After lane merging, up to 400 GB/sample
Maximum files uploaded per Alissa Reporter run	768 files	—
Read number allowance per file before subsampling	150M reads for <i>Human All Exon V7 Germline</i> or <i>Human All Exon V8 Germline</i> application	>150M reads are randomly subsampled to 150M reads

### Using Agilent’s AGeNT software for SureSelect XT HS2 DNA NGS workflows

Agilent’s AGeNT software is a Java-based toolkit used for SureSelect XT HS2 DNA library read processing steps. The AGeNT tools are 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](#).

The type of SureSelect HS2 adaptor used for sample library preparation determines the correct read pre-processing workflow for the sample, as summarized in [Table 48](#). Use of the AGeNT read processing tools is outlined briefly below. See the [AGeNT Best Practices](#) document sections covering the workflow suitable for your SureSelect libraries for more information.

**Table 48** Adaptor-based AGeNT workflow selection

HS2 Library Adaptor Type	Suitable AGeNT Workflow
MBC-free	SureSelect XT
MBC-tagged	SureSelect XT HS2

Prior to variant discovery, demultiplexed SureSelect XT HS2 library FASTQ data are pre-processed to remove sequencing adaptors and extract the MBC sequences (if present) using the AGeNT Trimmer module.

The trimmed reads should be aligned (and MBC tags added to the aligned BAM file where applicable) using a suitable tool such as BWA-MEM.

Once alignment and tagging are complete, the AGeNT CReaK (Consensus Read Kit) tool is used to generate consensus reads and mark or remove duplicates. The resulting BAM files are ready for downstream analysis including variant discovery.

#### 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](#) and review the FAQs for a detailed comparison of LocatIt and CReaK. LocatIt remains available for backward compatibility but CReaK is the recommended tool.

**NOTE**

If your libraries are MBC-tagged but 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 remaining read length after subtracting the 5 masked bases, e.g., use **N5Y146,I8,I8,N5Y146** for 2x150 NGS set up as shown in [Table 45](#) on page 48). The sum of the values following N and Y must match 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 **N5Y146;I8,I8;N5Y146** for 2x150 NGS set up as shown in [Table 45](#) on page 48). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

Alternatively, the first 5 bases may be trimmed from the demultiplexed FASTQ files using a suitable processing tool of your choice, such as seqtk. The AGeNT Trimmer module can also be used to remove the MBCs while trimming adaptor sequences. Non-Agilent adaptor trimmers will fail to remove the MBC sequences from the opposite adaptor (refer to [Figure 8](#)), which may affect alignment quality.

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## 6 Appendix: Using FFPE-derived DNA Samples

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This appendix provides a summary of the protocol modifications to apply to FFPE samples based on the integrity of the FFPE sample DNA.

## Protocol modifications for FFPE Samples

Protocol modifications that should be applied to FFPE samples are summarized in [Table 49](#).

**Table 49** Summary of protocol modifications for FFPE samples

Workflow Step and page	Parameter	Condition for non-FFPE Samples	Condition for FFPE Samples
gDNA Sample Preparation <a href="#">page 16</a>	Qualification of DNA Integrity	Not required	Required
DNA input for Library Preparation <a href="#">page 16</a>	Input amount and means of quantification	10 ng to 200 ng, quantified by Qubit assay	Based on determined DNA integrity (see <a href="#">Table 10</a> and <a href="#">Table 11</a> on page 17)
DNA Shearing <a href="#">page 18</a>	Mode of DNA Shearing	2 × 120 seconds (for 2 × 100 reads) 2 × 60 seconds (for 2 × 150 reads)	240 seconds (continuous, for all read lengths)
Pre-capture PCR <a href="#">page 28</a>	Cycle number	8–11	11–14
Sequencing <a href="#">page 55</a>	Output augmentation	Per project requirements	1× to 10× based on determined DNA integrity (see <a href="#">Table 50</a> and <a href="#">Table 51</a> on page 55)

## Methods for FFPE Sample Qualification

DNA integrity may be assessed using the Agilent NGS FFPE QC Kit or using the Agilent TapeStation system with the Genomic DNA ScreenTape.

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include the precise quantity of amplifiable DNA in the sample to allow direct normalization of input DNA amount and a  $\Delta\Delta Cq$  DNA integrity score used to design other protocol modifications.

The Agilent TapeStation instrument, combined with the Genomic DNA ScreenTape assay, provides an automated electrophoresis method for determination of a DNA Integrity Number (DIN) score used to estimate amount of input DNA required for sample normalization and to design other protocol modifications.

# Sequencing Output Recommendations for FFPE Samples

After determining the amount of sequencing output required for intact DNA samples to meet the goals of your project, use the guidelines below to determine the amount of extra sequencing output required for FFPE DNA samples.

**Samples qualified using  $\Delta\Delta\text{Cq}$ :** For samples qualified based on the  $\Delta\Delta\text{Cq}$  DNA integrity score, use the guidelines in [Table 50](#). For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with  $\Delta\Delta\text{Cq}$  score of 1 requires 200–400 Mb of sequencing output to achieve the same coverage.

**Table 50** Recommended sequencing augmentation for FFPE-derived DNA samples

$\Delta\Delta\text{Cq}$ value	Recommended fold increase for FFPE-derived sample
<0.5	No extra sequencing output
between 0.5 and 2	Increase sequencing allocation by 2x to 4x
>2	Increase sequencing allocation by 5x to 10x or more

**Samples qualified using DIN:** For samples qualified based on the Genomic DNA ScreenTape assay DIN integrity score, use the guidelines in [Table 51](#). For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with DIN score of 4 requires approximately 200–400 Mb of sequencing output to achieve the same coverage.

**Table 51** Recommended sequencing augmentation for FFPE-derived DNA samples

DIN value	Recommended fold increase for FFPE-derived sample
$\geq 8$	No extra sequencing output
between 3 and 8	Increase sequencing allocation by 2x to 4x
<3	Increase sequencing allocation by 5x to 10x or more

## 7 Reference

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This section contains reference information, including Reagent Kit contents, index sequences, troubleshooting information and a quick- reference protocol for experienced users.



# Reagent Kit Contents

## Kits for the Library Prep (+/-MBCs), Fast Hyb, Pre-capture Pooling Workflow

The SureSelect XT HS2 DNA workflow supported in this publication uses the kits listed in [Table 52](#). Detailed contents of each of the multi-part component kits listed in [Table 52](#) are shown in [Table 53](#) through [Table 57](#) on the following pages.

**Table 52** Kits Required for SureSelect XT HS2 Library Preparation with Fast Hyb/Pre-capture Pooling Workflow

SureSelect Library Type	Purchased Kits	Included Component Kits	Component Kit Part Numbers (specific index pairs included)	Storage Condition
<b>MBC-tagged HS2 DNA libraries</b> (96 Samples)	G9985A (Indexes 1-96), G9985B (Indexes 97-192), G9985C (Indexes 193-288), <b>OR</b> G9985D (Indexes 289-384)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR)	5500-0147	-20°C
		SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	5191-5688 (1-96), 5191-5689 (97-192), 5191-5690 (193-288), <b>OR</b> 5191-5691 (289-384)	-20°C
	G9987A (12 Hybridizations)	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	5191-6689	RT
		SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	5191-6690	-20°C
<b>MBC-free HS2 DNA libraries</b> (96 Samples)	G9956A (Indexes 1-96), G9956B (Indexes 97-192), G9956C (Indexes 193-288), <b>OR</b> G9956D (Indexes 289-384)	SureSelect XT HS2 Library Preparation Kit for ILM, MBC-Free (Pre PCR)	5282-0052	-20°C
		SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	5191-5688 (1-96), 5191-5689 (97-192), 5191-5690 (193-288), <b>OR</b> 5191-5691 (289-384)	-20°C
	G9987A (12 Hybridizations)	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	5191-6689	RT
		SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	5191-6690	-20°C

## Component Kit Details

**Table 53** SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR) content

Kit Component	96 Reaction Kit (p/n 5500-0147)
End Repair-A Tailing Enzyme Mix	tube with orange cap
End Repair-A Tailing Buffer	bottle
T4 DNA Ligase	tube with blue cap
Ligation Buffer	bottle
SureSelect XT HS2 Adaptor Oligo Mix	tube with white cap
Herculase II Fusion DNA Polymerase	tube with red cap
5x Herculase II Reaction Buffer with dNTPs	tube with clear cap

**Table 54** SureSelect XT HS2 Library Preparation Kit for ILM, MBC-Free (Pre PCR) content

Kit Component	96 Reaction Kit (p/n 5282-0052)
End Repair-A Tailing Enzyme Mix	tube with orange cap
End Repair-A Tailing Buffer	bottle
T4 DNA Ligase	tube with blue cap
Ligation Buffer	bottle
SureSelect MBC-Free Adaptor Oligo Mix	tube with black cap
Herculase II Fusion DNA Polymerase	tube with red cap
5x Herculase II Reaction Buffer with dNTPs	tube with clear cap

**Table 55** SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) content

Kit Component	p/n 5191-5688	p/n 5191-5689	p/n 5191-5690	p/n 5191-5691
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	Orange 96-well plate (index pairs 1–96)*	Blue 96-well plate (index pairs 97–192)*	Green 96-well plate (index pairs 193–288)*	Red 96-well plate (index pairs 289–384)*

\* See [page 60](#) through [page 68](#) for index pair sequence information; see [page 69](#) for index plate position maps.

**Table 56** SureSelect Target Enrichment Kit, ILM Hyb Module Box 1 (Post PCR) content

Kit Component	12 Hyb Kit (p/n 5191-6689)
SureSelect Binding Buffer	bottle
SureSelect Wash Buffer 1	bottle
SureSelect Wash Buffer 2	bottle

**Table 57** SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR) content

<b>Kit Component</b>	<b>12 Hyb Kit (p/n 5191-6690)</b>
SureSelect Fast Hybridization Buffer	bottle
SureSelect XT HS2 Blocker Mix	tube with blue cap
SureSelect RNase Block	tube with purple cap
SureSelect Post-Capture Primer Mix	tube with clear cap
Herculase II Fusion DNA Polymerase	tube with red cap
5x Herculase II Reaction Buffer with dNTPs	tube with clear cap

## SureSelect XT HS2 Index Primer Pair Information

The SureSelect XT HS2 Index Primer Pairs are provided pre-combined. Each member of the primer pair contains a unique 8-bp P5 or P7 index, resulting in dual-indexed NGS libraries. One primer pair is provided in each well of a 96-well plate (see [page 69](#) to [page 70](#) for plate maps). Each well contains a single-use aliquot of a specific pair of P7 plus P5 primers.

### CAUTION

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

The nucleotide sequence of the index portion of each primer is provided in [Table 59](#) on page 61 through [Table 66](#) on page 68. 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 58](#). 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 58** 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.

## SureSelect XT HS2 Index Primer Pair Sequences

Table 59 SureSelect XT HS2 Index Primer Pairs 1–48, provided in orange 96-well plate or in strip tubes

Primer Pair #	Index Strip	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Index Strip	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	GCAGGTTC	AGAAGCAA	TTGCTTCT	31	G04	GCAACAAT	TCAGAGGT	ACCTCTGA
8	H01	AAGTGTCT	GCAGGTTC	GAACCTGC	32	H04	GTGATCG	GCAACAAT	ATTGTTGC
9	A02	CTACCGAA	AAGTGTCT	AGACACTT	33	A05	ATGGTAGC	GTGATCG	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	GTTTCAT
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 60** SureSelect XT HS2 Index Primer Pairs 49–96, provided in orange 96-well plate

Primer Pair #	Index Strip	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Index Strip	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	AGGTTCTT
56	H07	CAGCTTGC	GACTCAAT	ATTGAGTC	80	H10	TACGTAGC	CTGTGCCT	AGGCACAG
57	A08	AGCTGAAG	CAGCTTGC	GCAAGCTG	81	A11	AAGTGGAC	TACGTAGC	GCTACGTA
58	B08	ATTCCGTG	AGCTGAAG	CTTCAGCT	82	B11	CAACCGTG	AAGTGGAC	GTCCACTT
59	C08	TATGCCGC	ATTCCGTG	CACGGAAT	83	C11	CTGTTGTT	CAACCGTG	CACGGTTG
60	D08	TCAGCTCA	TATGCCGC	GCGGCATA	84	D11	GCACGATG	CTGTTGTT	AACAACAG
61	E08	AACTGCAA	TCAGCTCA	TGAGCTGA	85	E11	GTACGGAC	GCACGATG	CATCGTGC
62	F08	ATTAGGAG	AACTGCAA	TTGCAGTT	86	F11	CTCCAAGC	GTACGGAC	GTCCGTAC
63	G08	CAGCAATA	ATTAGGAG	CTCCTAAT	87	G11	TAGTCTGA	CTCCAAGC	GCTTGGAG
64	H08	GCCAAGCT	CAGCAATA	TATTGCTG	88	H11	TTCGCCGT	TAGTCTGA	TCAGACTA
65	A09	TCCGTTAA	GCCAAGCT	AGCTTGGC	89	A12	GAACTAAG	ATACGAAG	CTTCGTAT
66	B09	GTGCAACG	TCCGTTAA	TTAACGGA	90	B12	AAGCCATC	GAGATTCA	TGAATCTC
67	C09	AGTAACGC	GTGCAACG	CGTTGCAC	91	C12	AACTCTTG	AAGCCATC	GATGGCTT
68	D09	CATAGCCA	AGTAACGC	GCGTFACT	92	D12	GTAGTCAT	AACTCTTG	CAAGAGTT
69	E09	CACTAGTA	CATAGCCA	TGGCTATG	93	E12	CTCGCTAG	GTAGTCAT	ATGACTAC
70	F09	TTAGTGCG	CACTAGTA	TACTAGTG	94	F12	AGTCTTCA	CAGTATCA	TGATACTG
71	G09	TCGATACA	TTAGTGCG	CGCACTAA	95	G12	TCAAGCTA	CTTCGTAC	GTACGAAG
72	H09	ATAGTGAC	TCGATACA	TGTATCGA	96	H12	CTTATCCT	TCAAGCTA	TAGCTTGA

**Table 61** SureSelect XT HS2 Index Primer Pairs 97–144, provided in blue 96-well plate

Primer Pair #	Index Strip	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Index Strip	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	GTTGTCGA	133	E05	AGTTGCCG	GATGCCAA	TTGGCATC
110	F02	CAAGGAAG	TCGATGTT	AACATCGA	134	F05	GTCCACCT	AGTTGCCG	CGGCAACT
111	G02	ATTGATGC	AGAGAATC	GATTCTCT	135	G05	ATCAAGGT	GTCCACCT	AGGTGGAC
112	H02	TCGCAGAT	TTGATGGC	GCCATCAA	136	H05	GAACCAGA	ATCAAGGT	ACCTTGAT
113	A03	GCAGAGAC	TCGCAGAT	ATCTGCGA	137	A06	CATGTTCT	GAACCAGA	TCTGGTTC
114	B03	CTGCGAGA	GCAGAGAC	GTCTCTGC	138	B06	TCACTGTG	CATGTTCT	AGAACATG
115	C03	CAACCAAC	CTGCGAGA	TCTCGCAG	139	C06	ATTGAGCT	TCACTGTG	CACAGTGA
116	D03	ATCATGCG	CAACCAAC	GTTGGTTG	140	D06	GATAGAGA	ATTGAGCT	AGCTCAAT
117	E03	TCTGAGTC	ATCATGCG	CGCATGAT	141	E06	TCTAGAGC	GATAGAGA	TCTCTATC
118	F03	TCGCCTGT	TCTGAGTC	GACTCAGA	142	F06	GAATCGCA	TCTAGAGC	GCTCTAGA
119	G03	GCGCAATT	TCGCCTGT	ACAGGCGA	143	G06	CTTCACGT	GAATCGCA	TGCGATTG
120	H03	AGACGCCT	GCGCAATT	AATTGCGC	144	H06	CTCCGTT	CTTCACGT	ACGTGAAG

**Table 62** SureSelect XT HS2 Index Primer Pairs 145–192, provided in blue 96-well plate

Primer Pair #	Index Strip	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Index Strip	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
145	A07	TGTGACTA	CTCCGGTT	AACCGGAG	169	A10	CGCTCAGA	CTAACAAG	CTTGTTAG
146	B07	GCTTCCAG	TGTGACTA	TAGTCACA	170	B10	TAACGACA	CGCTCAGA	TCTGAGCG
147	C07	CATCCTGT	GCTTCCAG	CTGGAAGC	171	C10	CATACTTG	TAACGACA	TGTCGTTA
148	D07	GTAATACG	CATCCTGT	ACAGGATG	172	D10	AGATACGA	CATACTTG	CAAGTATG
149	E07	GCCAACAA	GTAATACG	CGTATTAC	173	E10	AATCCGAC	AGATACGA	TCGTATCT
150	F07	CATGACAC	GCCAACAA	TTGTTGGC	174	F10	TGAAGTAC	AATCCGAC	GTCGGATT
151	G07	TGCAATGC	CATGACAC	GTGTCATG	175	G10	CGAATCAT	TGAAGTAC	GTACTIONA
152	H07	CACATTCG	TGCAATGC	GCATTGCA	176	H10	TGATTGGC	CGAATCAT	ATGATTTCG
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	TGTTTCGCG
157	E08	GAGTAAGA	ATAGCGTT	AACGCTAT	181	E11	AGAACCGT	TACGGTTG	CAACCGTA
158	F08	CTGACACA	GAGTAAGA	TCTTACTC	182	F11	AGGTGCTT	AGAACCGT	ACGGTTCT
159	G08	ATACGTGT	CTGACACA	TGTGTCAG	183	G11	ATCGCAAC	AGGTGCTT	AAGCACCT
160	H08	GACCGAGT	ATACGTGT	ACACGTAT	184	H11	GCCTCTCA	ATCGCAAC	GTTGCGAT
161	A09	GCAGTTAG	GACCGAGT	ACTCGGTC	185	A12	TCGCGTCA	GCCTCTCA	TGAGAGGC
162	B09	CGTTCGTC	GCAGTTAG	CTAACTGC	186	B12	GAGTGCGT	TCGCGTCA	TGACGCGA
163	C09	CGTTAACG	CGTTCGTC	GACGAACG	187	C12	CGAACACT	GCATAAGT	ACTTATGC
164	D09	TCGAGCAT	CGTTAACG	CGTTAACG	188	D12	TAAGAGTG	AGAAGACG	CGTCTTCT
165	E09	GCCGTAAC	TCGAGCAT	ATGCTCGA	189	E12	TGGATTGA	TAAGAGTG	CACTCTTA
166	F09	GAGCTGTA	GCCGTAAC	GTTACGGC	190	F12	AGGACATA	TGGATTGA	TCAATCCA
167	G09	AGGAAGAT	GAGCTGTA	TACAGCTC	191	G12	GACATCCT	AGGACATA	TATGTCCT
168	H09	CTAACAAG	AGGAAGAT	ATCTTCCT	192	H12	GAAGCCTC	GACATCCT	AGGATGTC



**Table 63 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	AAGTGACT	219	C04	AGGTGAGA	TCTATGCG	CGCATAGA
196	D01	TCAGACAA	AGCATACA	TGTATGCT	220	D04	CACAACTT	AGGTGAGA	TCTCACCT
197	E01	TTGGAGAA	TCAGACAA	TTGTCTGA	221	E04	TTGTGTAC	CACAACTT	AAGTTGTG
198	F01	TTAACGTG	TTGGAGAA	TTCTCAA	222	F04	TCACAAGA	TTGTGTAC	GTACACAA
199	G01	CGTCTGTG	TTAACGTG	CACGTTAA	223	G04	GAAGACCT	TCACAAGA	TCTTGTGA
200	H01	AACCTAAC	CGTCTGTG	CACAGACG	224	H04	AGTTCTGT	GAAGACCT	AGGTCTTC
201	A02	AGAGTGCT	AACCTAAC	GTTAGGTT	225	A05	GCAGTGTT	AGTTCTGT	ACAGAACT
202	B02	TTATCTCG	AGAGTGCT	AGCACTCT	226	B05	AGGCATGC	GCAGTGTT	AACACTGC
203	C02	CATCAGTC	TTATCTCG	CGAGATAA	227	C05	AAGGTACT	AGGCATGC	GCATGCCT
204	D02	AAGCACAA	CATCAGTC	GACTIONG	228	D05	CACTAAGT	AAGGTACT	AGTACCTT
205	E02	CAGTGAGC	AAGCACAA	TTGTGCTT	229	E05	GAGTCCTA	CACTAAGT	ACTTAGTG
206	F02	GTCGAAGT	CAGTGAGC	GCTCACTG	230	F05	AGTCCTTC	GAGTCCTA	TAGGACTC
207	G02	TCTCATGC	GTCGAAGT	ACTTCGAC	231	G05	TTAGGAAC	AGTCCTTC	GAAGGACT
208	H02	CAGAAGAA	TCTCATGC	GCATGAGA	232	H05	AAGTCCAT	TTAGGAAC	GTTCCATA
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	GAGCATAAC	GTACCTTG	CAAGGTAC
216	H03	CACGAGCT	ATCTACCA	TGGTAGAT	240	H06	TGCTTACG	GAGCATAAC	GTATGCTC

**Table 64 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	TGCTTACG	CGTAAGCA	265	A10	CAATGCTG	CATGAATG	CATTCATG
242	B07	TAGCTATG	AAGAGACA	TGTCTCTT	266	B10	CTTGATCA	CAATGCTG	CAGCATTG
243	C07	TCTGCTAC	TAGCTATG	CATAGCTA	267	C10	GCGAATTA	CTTGATCA	TGATCAAG
244	D07	GTCACAGA	TCTGCTAC	GTAGCAGA	268	D10	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	TCCGAACT	TCATACCG	CGGTATGA	272	H10	GTTGCAAG	ATAACGGC	GCCGTTAT
249	A08	AGAGAGAA	TCCGAACT	AGTTCGGA	273	A11	AGCCGGAA	GTTGCAAG	CTTGCAAC
250	B08	GATCGTTA	AGAGAGAA	TTCTCTCT	274	B11	AACAGCCG	AGCCGGAA	TTCCGGCT
251	C08	GCGCTAGA	GATCGTTA	TAACGATC	275	C11	CTAGTGTA	AACAGCCG	CGGCTGTT
252	D08	ATGACTCG	GCGCTAGA	TCTAGCGC	276	D11	GAGGCTCT	CTAGTGTA	TACTACTAG
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	TCGTTGTC	286	F12	GCTCTCTC	AAGTACTC	GAGTACTT
263	G09	TCTCCTTG	ATACTGCA	TGCAGTAT	287	G12	CTACCAGT	GCTCTCTC	GAGAGAGC
264	H09	CATGAATG	TCTCCTTG	CAAGGAGA	288	H12	GATGAGAT	CTACCAGT	ACTGGTAG

**Table 65 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	ATGTGAG	326	F05	AGGTCAT	CGAGTCGA	TCGACTCG
303	G02	CGGTCTCT	GAGATCGC	GCGATCTC	327	G05	GACGTGCA	AGGTCAT	ATGAGCCT
304	H02	TAACAC	CGGTCTCT	AGAGACCG	328	H05	GAACATGT	GACGTGCA	TGCACGTC
305	A03	CACAATGA	TAACAC	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 66 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	GTTTCATTC	GTCGGTAA	TTACCGAC	367	G10	GCATGCCT	CGTAACTG	CAGTTACG
344	H07	AAGCAGTT	GTTTCATTC	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	AACGTAAT	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 Plate Maps

**Table 67 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 68 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	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

**Table 69** 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 70** 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 recovery of gDNA from samples is low

- ✓ Using excess tissue for gDNA isolation can reduce yield. Use only the amount of each specific tissue type recommended by the gDNA isolation protocol.
- ✓ Tissue sample lysis may not have been optimal during gDNA isolation. Monitor the extent of sample lysis during the Proteinase K digestion at 56°C by gently pipetting the digestion reaction every 20–30 minutes, visually inspecting the solution for the presence of tissue clumps. If clumps are still present after the 1-hour incubation at 56°C, add another 10 µL of Proteinase K and continue incubating at 56°C, with periodic mixing and visual inspections, for up to two additional hours. When the sample no longer contains clumps of tissue, move the sample to room temperature until lysis is complete for the remaining samples. Do not over-digest. Individual samples may be kept at room temperature for up to 2 hours before resuming the protocol. Do not exceed 3 hours incubation at 56°C for any sample.

## If concentration of DNA samples is too low for enzymatic fragmentation

- ✓ The standard enzymatic fragmentation protocol requires 10–200 ng DNA in a volume of 7 µL, and uses a final fragmentation reaction volume of 10 µL. For dilute samples, enzymatic fragmentation may be performed in a reaction volume of 20 µL using the modified protocol below:
  - Bring samples containing 10–200 ng DNA to 17 µL final volume with 1X Low TE Buffer in plate or strip sample wells.
  - Prepare the Fragmentation master mix as directed in [Table 16](#) on [page 20](#).
  - Add 3 µL of the master mix to each DNA sample well. Mix and spin as directed on [page 21](#).
  - Run the thermal cycling program in [Table 14](#) on [page 20](#) using the 37°C fragmentation duration shown in the table below.

NGS read length	High-quality DNA samples	FFPE DNA samples
2 × 100 reads	25 minutes	25 minutes
2 × 150 reads	15 minutes	25 minutes

## 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 24](#)) 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. If a high molecular weight peak (>500 bp) is observed in the electropherogram for a sample with low yield, the DNA may be overamplified. Repeat library preparation for the sample, decreasing the pre-capture PCR cycle number by 1 to 3 cycles.
- ✓ DNA isolated from FFPE tissue samples may be over-fragmented or have modifications that adversely affect library preparation processes. Use the Agilent NGS FFPE QC Kit to determine the precise quantity of amplifiable DNA in the sample and allow direct normalization of input DNA amount.
- ✓ Yield from the AMPure XP Bead purification steps may be suboptimal. Consider the factors below:
  - Adhere to all of the bead and reagent handling steps in the protocol. In particular, make sure to equilibrate beads at room temperature for at least 30 minutes before use, and use freshly-prepared 70% ethanol (prepared on day of use) for each purification procedure.

- Ensure that the AMPure XP Beads are not over-dried just prior to sample elution (see [step 12 on page 27](#) and [page 30](#)). If you are drying the beads at 37°C for 2 minutes, consider using drying conditions of RT for 5 minutes.
- Increasing the elution incubation time (up to 10 minutes) may improve recovery, especially for longer DNA fragments.

### **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 sheared DNA pre-capture library fragment size is larger than expected in electropherograms**

- ✓ Shearing may not be optimal. For intact, high-quality DNA samples, ensure that shearing is completed using the two-round shearing protocol provided, including all spinning and vortexing steps.
- ✓ Any bubbles present on the microTUBE filament may disrupt complete shearing. Spin the microTUBE for 30 seconds before the first round of shearing to ensure that any bubbles are released.

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

- ✓ FFPE DNA pre-capture libraries may have a smaller fragment size distribution due to the presence of DNA fragments in the sample input that are smaller than the targeted post-fragmentation size. Adhere to the DNA quality guidelines provided on [page 16](#).
- ✓ 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 29](#).

### **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 32](#). 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 25](#). In particular, ensure that the ligation master mix is mixed with the sample prior to adding the 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.
- ✓ For whole-genome sequencing (not specifically supported by this protocol), samples with an adaptor-dimer peak must be subjected to an additional round of SPRI-purification. To complete, first dilute the sample to 50 µL with nuclease free water, then follow the SPRI purification procedure on [page 29](#).
- ✓ When preparing MBC-free RNA sequencing libraries, the SureSelect MBC-Free Adaptor Oligo Mix provided in this kit should be diluted 5-fold before use (see [page 23](#)). Use of the undiluted adaptor solution may increase the quantity of adaptor-dimers in the cDNA library.

### **If vacuum concentrator is not available for pre-capture library volume reduction prior to hybridization**

- ✓ The standard hybridization protocol requires 12 µL samples containing 1.5 or 3 µg of pooled library DNA, using a vacuum concentrator to reduce the volume of the library pools where needed. If a vacuum concentrator is not available in your laboratory, use the protocol modifications below to generate concentrated library pools for hybridization. This modification may result in some loss of library complexity.



- Pool indexed libraries to contain 2.5X amount of each library DNA given in [Table 30](#) on page 34. For example, for the SureSelect XT HS PreCap Human All Exon V8 probe, pool 937.5 ng of each of 8 indexed libraries for total pool of 7.5 µg library DNA.
- Concentrate each pool using an additional round of AMPure XP Bead purification. Follow the AMPure XP Bead purification instructions on [page 29](#) to [page 30](#), modifying [step 3](#) to use a bead volume of 1.8X the total pool volume. Elute in 24 µL of nuclease-free water in [step 13](#), then place 12 µL of each concentrated pool in a separate well for the hybridization reaction.

### 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 probe 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 probe hybridization mix is prepared immediately before use, as directed on [page 37](#), and that solutions containing the probe are not held at room temperature for extended periods.
- ✓ Yield from the AMPure XP Bead purification step may be suboptimal. Consider the factors below:
  - Adhere to all of the bead and reagent handling steps in the protocol. In particular, make sure to equilibrate beads at room temperature for at least 30 minutes before use, and use freshly-prepared 70% ethanol (prepared on day of use) for each purification procedure.
  - Ensure that the AMPure XP Beads are not over-dried just prior to sample elution (see [step 12](#) on [page 42](#)). If you are drying the beads at 37°C for 2 minutes, consider using drying conditions of RT for 5 minutes.
  - Increasing the elution incubation time (up to 10 minutes) may improve recovery, especially for longer DNA fragments.

### 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 41](#).

### If low percent 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 38](#)). 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 39](#))
  - Bead suspensions are mixed thoroughly during washes by pipetting up and down and vortexing (see [page 39](#))
- ✓ Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a vortex and plate spinner or centrifuge in close proximity to thermal cycler to retain the 65°C sample temperature during mixing and transfer steps ([step 7](#) to [step 8](#) on [page 37](#)).

## 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 SureSelect XT HS Human All Exon V8-based probes or SureSelect XT HS Clinical Research Exome V4 using the hybridization program in [Table 31](#) on [page 35](#) (including segment with one-hour incubation at 65°C), repeat target enrichment 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 32](#) on [Table 32](#)).
- ✓ Redesign custom target enrichment probes designed for the XT platform employing SureDesign's XT HS-boosting parameters during design. Alternatively, consider switching to use of the Overnight Hyb/Pre-cap Pooling workflow detailed in publication [G9957-90000](#) when using probes designed for the XT platform.

# Quick Reference Protocol

An abbreviated summary of the protocol steps is provided below for experienced users. Use the complete protocol on [page 16](#) to [page 44](#) until you are familiar with all of the protocol details such as reagent mixing instructions and instrument settings.

Step	Summary of Conditions
<b>Library Prep</b>	
Prepare, qualify, and fragment DNA samples	Prepare 10–200 ng gDNA in Low TE (50 µL for Covaris/7 µL for enzymatic fragmentation). For FFPE DNA, qualify integrity and adjust input amount as directed on <a href="#">page 16</a> and <a href="#">page 17</a> . Mechanically shear DNA using Covaris with shearing conditions on <a href="#">page 19</a> OR enzymatically fragment DNA using SureSelect Enzymatic Fragmentation Kit with protocol on <a href="#">page 20</a> – <a href="#">page 21</a> . Either method yields fragmented DNA in final sample volume of 50 µL.
Prepare Ligation master mix	Per 96 reactions: 2300 µL Ligation Buffer + 200 µL T4 DNA Ligase Keep at room temperature 30–45 min before use
Prepare End-Repair/dA-Tailing master mix	Per 96 reactions: 1600 µL End Repair-A Tailing Buffer + 400 µL End Repair-A Tailing Enzyme Mix Keep on ice
End-Repair and dA-Tail the DNA fragments	50 µL DNA 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 Adaptor Oligo Mix <b>OR</b> 5 µL SureSelect XT HS2 MBC-Free Adaptor Oligo Mix Incubate in thermal cycler: 30 min @ 20°C, Hold @ 4°C
Purify DNA	100 µL DNA sample + 80 µL AMPure XP bead suspension Elute DNA in 35 µL nuclease-free H <sub>2</sub> O, removing 34 µL to fresh well
Prepare PCR master mix	Per 96 reactions: 1000 µL 5× Herculase II Reaction Buffer with dNTPs + 100 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 28</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>	
Prep DNA in hyb plate	In wells of hybridization plate or strip, pool equal amounts of 8 or 16 indexed libraries per well for total of 1.5 or 3 µg library DNA per pool (depending on probe design—see <a href="#">Table 30</a> on page 34). Adjust volume to 12 µL with nuclease-free H <sub>2</sub> O (and using vacuum concentrator at <45°C where needed).
Program thermal cycler	Input the appropriate thermal cycler program on <a href="#">page 35</a> and pause program
Run pre-hybridization blocking protocol	12 µL library DNA + 5 µL SureSelect XT HS2 Blocker Mix Run paused thermal cycler program segments 1 through 3 Keep at 65°C hold in segment 3 until Hyb Mix (described below) added

Step	Summary of Conditions
Prepare Hyb Mix	Prepare 25% RNase Block dilution, then prepare appropriate Probe Hyb Mix below (amounts shown are for 12 hybridizations): Probes $\geq 3$ Mb: 26 $\mu$ L 25% RNase Block + 65 $\mu$ L Probe+ 78 $\mu$ L SureSelect Fast Hybridization Buffer Probes $< 3$ Mb: 26 $\mu$ L 25% RNase Block + 26 $\mu$ L Probe + 39 $\mu$ L nuclease-free H <sub>2</sub> O + 78 $\mu$ L SureSelect Fast Hybridization Buffer
Run the hybridization	With cyclor held in segment 3 and samples retained in cyclor, add 13 $\mu$ L Probe Hyb Mix to wells Resume the thermal cyclor program, completing the remaining hybridization segment(s) and final 65°C or 21°C hold segment
Prepare streptavidin beads	Wash 50 $\mu$ L Streptavidin T1 beads 3 $\times$ in 200 $\mu$ L SureSelect Binding Buffer
Capture hybridized libraries	Add hybridized samples (~30 $\mu$ L) to washed streptavidin beads (200 $\mu$ L) Incubate 30 min at RT with vigorous shaking (1400-1900 rpm) During incubation, pre-warm 6 $\times$ 200 $\mu$ L 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 $\times$ with 200 $\mu$ L SureSelect Wash Buffer 1 at RT Wash beads 6 $\times$ with 200 $\mu$ L pre-warmed SureSelect Wash Buffer 2 (5 minutes at 70°C per wash) Resuspend washed beads in 25 $\mu$ L nuclease-free H <sub>2</sub> O
<b>Post-capture amplification</b>	
Prepare PCR master mix	Per 12 reactions: 182 $\mu$ L nuclease-free H <sub>2</sub> O + 140 $\mu$ L 5 $\times$ Herculase II Reaction Buffer with dNTPs + 14 $\mu$ L SureSelect Post-Capture Primer Mix + 14 $\mu$ 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 cyclor using conditions on <a href="#">page 40</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

## In This Book

This guide provides instructions for using SureSelect HS2 DNA Reagent Kits with a Fast-Hyb, pre-capture pooling workflow.

