SureSelect XT HS2 DNA System

DNA Library Preparation and Target Enrichment for the Illumina Platform

Protocol

Version D0, April 2021

SureSelect platform manufactured with Agilent SurePrint Technology

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Agilent Technologies

Notices

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In this Guide...

This guide provides an optimized protocol for preparation of target-enriched Illumina paired-end multiplexed sequencing libraries using the SureSelect XT HS2 DNA system.

1 Before You Begin

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

2 Preparation and Fragmentation of Input DNA

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

3 Library Preparation

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

4 Hybridization and Capture

This chapter describes the steps to hybridize and capture the prepared DNA library using a SureSelect or ClearSeq Probe Capture Library.

5 Post-Capture Sample Processing for Multiplexed Sequencing

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

6 Appendix: Using FFPE-derived DNA Samples

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

7 Reference

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

What's New in Version D0

- Support for SureSelect XT HS Human All Exon V8 Probe. See Table 3 on page 14 for ordering information and see Table 28 on page 51 for the hybridization thermal cycling program recommended for this probe with the SureSelect XT HS2 DNA system. Also see troubleshooting information on page 101 and updates to the *Quick Reference Protocol* on page 103.
- Updates to instructions in the "Hybridization and Capture" chapter on page 49 through page 56. Updates include provision of two separate hybridization thermal cycler programs (Table 28 and Table 29 on page 51) and related changes throughout the chapter.
- New footnotes to Table 11 on page 25 and Table 14 on page 28 on FFPE sample initial fragment size impacts on library fragment size distribution.
- Update to Figure 4 on page 46 and associated text on page 47.
- Update to downstream sequencing support information in Table 41 on page 71.
- Update to description of flat strip caps in Table 8 on page 19.

What's New in Version C1

- Updates to index pair sequence tables (page 87 through page 94) including updates to P5 index platform descriptions and correction of well position typographical errors
- Updates to downstream sequencing support information (see Table 41 on page 71 and *Note* on page 86)
- Updates to molecular barcode and associated dark base information (see Figure 8 on page 70) and instructions for processing using the Agilent Genomics NextGen Toolkit (see page 72)

- Addition of hybridization temperature considerations for probes designed for use with the SureSelect XT system (see Table 28 on page 51)

What's New in Version C0

- Support for revised SureSelect custom probe products, produced using an updated manufacturing process beginning August, 2020 (see Table 3 on page 14). Custom probes produced using the legacy manufacturing process are also fully supported by the protocols in this document. Probe information was reorganized (see Table 3 on page 14), and probe nomenclature throughout document was updated.
- Updates to DNA library quantitation/qualification guidelines including increased support for the Agilent's Fragment Analyzer platform and streamlined sample analysis guidelines (see Table 6 on page 17 and see page 44 through page 46 and page 65 through page 67).
- Updates to thermal cycler and plasticware recommendations and usage instructions (see *Caution* and Table 5 on page 16 and see *Note* on page 34).
- Updates to *Materials Required* including updated ordering information for Dynabeads MyOne Streptavidin T1 beads (Table 4 on page 15) and for Eppendorf ThermoMixer C and Qubit Fluorometer (Table 5 on page 16).
- Updates to *Optional Materials* in Table 8 on page 19, including removal of ethylene glycol supplier information (see page 25 for related update to DNA shearing set up instructions).
- Updates to "SureSelect XT HS2 Index Primer Pair Information" section (page 86 through page 97) including addition of P5 primer sequences oriented for the NextSeq, HiSeq 4000 and HiSeq 3000 platforms and reorganization of section content.

Content

1 Before You Begin

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

NOTE This protocol differs from the Illumina Multiplexed Paired-End sequencing manual and other SureSelect protocols at several steps. Pay close attention to the primers used for each amplification step and the blocking agents used during hybridization.

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

Overview of the Workflow

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

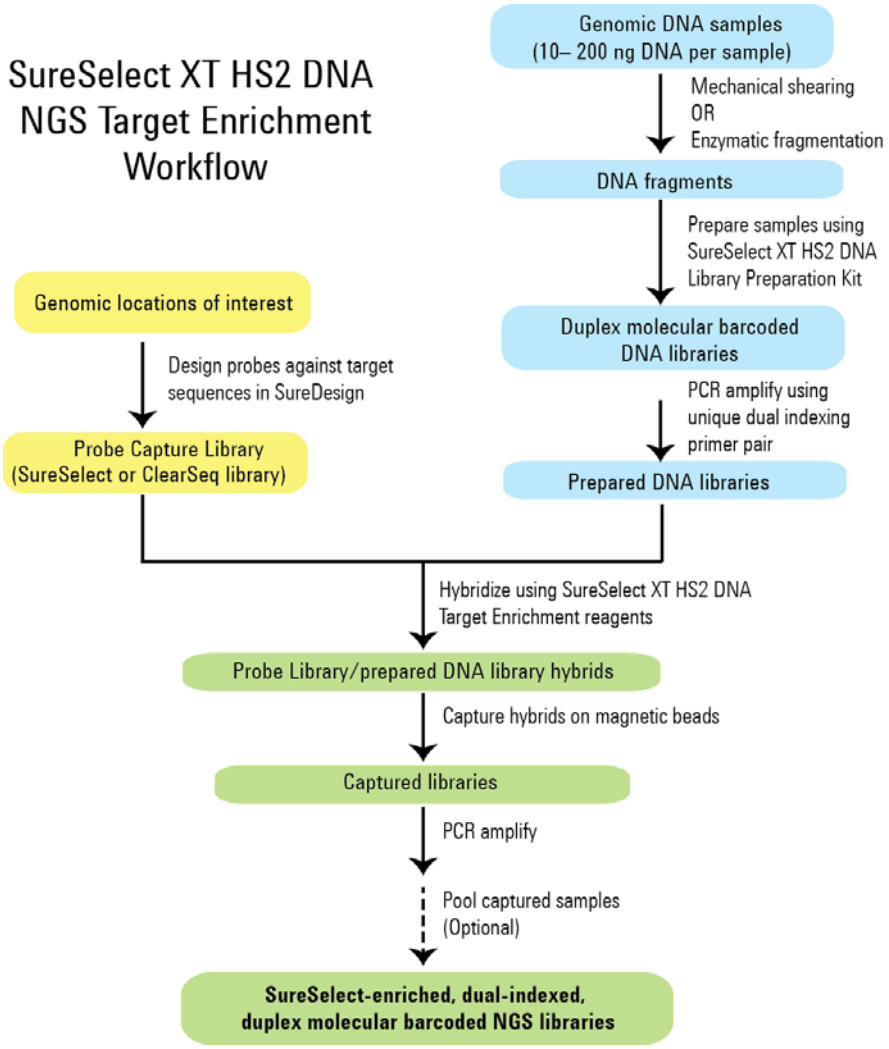


Figure 1 Overall target-enriched sequencing sample preparation workflow.

Table 1 Estimated time requirements (up to 16 sample run size)

Step	Time
Library Preparation	3.5 hours
Hybridization and Capture	3.5 hours
Post-capture amplification	1 hour
QC using Bioanalyzer or TapeStation platform and sample pooling	1.5 hours

Procedural Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Use best-practices to prevent PCR product 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 pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution, or equivalent.
  3. Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
  4. Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.
- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of 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 –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.

Safety Notes

CAUTION

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

Materials Required

Materials required to complete the SureSelect XT HS2 protocol will vary based on the following considerations:

- SureSelect XT HS2 DNA Reagent Kit format preference, where some options include ancillary reagent modules
- 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

To determine the materials required for your unique needs, first select the preferred kit format from Table 2 below and a compatible target enrichment probe from Table 3. Then refer to Table 4 through Table 7 for additional materials needed to complete the protocols using the selected kit format/DNA sample type/fragmentation method.

Table 2 SureSelect XT HS2 DNA Reagent Kit Varieties

Description	Kit Part Number	
	16 Reaction Kit*	96 Reaction Kit†
SureSelect XT HS2 DNA Reagent Kit	G9981A (with Index Pairs 1–16)	G9983A (with Index Pairs 1–96) G9983B (with Index Pairs 97–192) G9983C (with Index Pairs 193–288) G9983D (with Index Pairs 289–384)
Reagent Kits with additional component modules		
SureSelect XT HS2 DNA Starter Kit Includes the following modules: SureSelect XT HS2 DNA Reagent Kit SureSelect Enzymatic Fragmentation Kit SureSelect DNA AMPure® XP Beads‡ SureSelect Streptavidin Beads	G9982A (with Index Pairs 1–16)	Not applicable
SureSelect XT HS2 DNA Reagent Kit with AMPure® XP/Streptavidin Beads	Not applicable	G9984A (with Index Pairs 1–96) G9984B (with Index Pairs 97–192) G9984C (with Index Pairs 193–288) G9984D (with Index Pairs 289–384)

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

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

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

Table 3 Compatible Probes

Probe Capture Library	16 Reactions	96 Reactions
Pre-designed Probes		
SureSelect XT HS Human All Exon V8	5191-6873	5191-6874
SSel XT HS and XT Low Input Human All Exon V7	5191-4028	5191-4029
SureSelect XT Clinical Research Exome V2	5190-9491	5190-9492
SureSelect XT Mouse All Exon	5190-4641	5190-4642
ClearSeq Comprehensive Cancer XT	5190-8011	5190-8012
ClearSeq Inherited Disease XT	5190-7518	5190-7519
Custom Probes*		
SureSelect Custom Tier1 1–499 kb	Please visit the SureDesign website to design Custom SureSelect probes and obtain ordering information. Contact the SureSelect support team (see page 2) or your local representative if you need assistance. Custom probes are also available in a 480 Reaction package.	
SureSelect Custom Tier2 0.5 –2.9 Mb		
SureSelect Custom Tier3 3 –5.9 Mb		
SureSelect Custom Tier4 6 –11.9 Mb		
SureSelect Custom Tier5 12–24 Mb		
Pre-designed Probes customized with additional *Plus* custom content		
SSel XT HS and XT Low Input Human All Exon V7 Plus 1	Please visit the SureDesign website to design the customized *Plus* content and obtain ordering information. Contact the SureSelect support team (see page 2) or your local representative if you need assistance.	
SSel XT HS and XT Low Input Human All Exon V7 Plus 2		
SureSelect XT Clinical Research Exome V2 Plus 1		
SureSelect XT Clinical Research Exome V2 Plus 2		
ClearSeq Comprehensive Cancer Plus XT		
ClearSeq Inherited Disease Plus XT		

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

Table 4 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 500 assays	Thermo Fisher Scientific p/n Q32850 p/n Q32853	—
Nuclease-free Water	Thermo Fisher Scientific p/n AM9930	Water should not be DEPC-treated
AMPure® XP Kit 5 ml 60 ml 450 ml	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882	Separate purchase **not** required for use with SureSelect XT HS2 DNA Reagent Kits that include SureSelect DNA AMPure® XP Beads and SureSelect Streptavidin Beads (Agilent p/n G9982A, G9984A, G9984B, G9984C, or G9984D)
Dynabeads MyOne Streptavidin T1 2 ml 10 ml 50 ml	Thermo Fisher Scientific p/n 65601 p/n 65602 p/n 65604D	

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

Table 5 Required Equipment--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, domed	Consult the thermal cycler manufacturer's recommendations
Nucleic acid analysis system (instrument and consumables)	Select one system from Table 6 on page 17
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*
Multichannel pipette	Rainin Pipet-Lite Multi Pipette or equivalent
Single channel pipettes (10-, 20-, 200-, and 1000-µl capacity)	Rainin Pipet-Lite Pipettes or equivalent
Sterile, nuclease-free aerosol barrier pipette tips	general laboratory supplier
Vortex mixer	general laboratory supplier
Ice bucket	general laboratory supplier
Powder-free gloves	general laboratory supplier

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

Table 6 Nucleic Acid Analysis Platform Options--Select One

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

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

Description	Vendor and Part Number	Notes
Required for preparation of high-quality DNA samples (not required for FFPE DNA sample preparation)		
High-quality gDNA purification system, for example:		—
QIAamp DNA Mini Kit 50 Samples 250 Samples	Qiagen p/n 51304 p/n 51306	
Required for preparation of FFPE DNA samples (not required for high-quality DNA sample preparation)		
QIAamp DNA FFPE Tissue Kit, 50 Samples	Qiagen p/n 56404	—
Deparaffinization Solution	Qiagen p/n 19093	—
FFPE DNA integrity assessment system:		—
Agilent NGS FFPE QC Kit 16 reactions 96 reactions	Agilent p/n G9700A p/n G9700B	
OR		
TapeStation Genomic DNA Analysis Consumables: Genomic DNA ScreenTape Genomic DNA Reagents	Agilent p/n 5067-5365 p/n 5067-5366	
Required for mechanical shearing of DNA samples (not required for workflows with enzymatic fragmentation)		
Covaris Sample Preparation System	Covaris model E220	
Covaris microTUBE sample holders	Covaris p/n 520045	
Required for enzymatic fragmentation of DNA samples (not required for workflows with mechanical shearing)		
SureSelect Enzymatic Fragmentation Kit	Agilent p/n 5191-4079 (16 reactions) p/n 5191-4080 (96 reactions)	Not required for use with SureSelect XT HS2 DNA Starter Kit (Agilent p/n G9982A)

Optional Materials

Table 8 Supplier Information for Optional Materials

Description	Vendor and Part Number	Purpose
Tween 20	Sigma-Aldrich p/n P9416-50ML	Sequencing library storage (see page 69)
8× flat strip caps	Consult the thermal cycler manufacturer's recommendations	Sealing wells for protocol steps outside of hybridization/capture*
MicroAmp Clear Adhesive Film	Thermo Fisher Scientific p/n 4311971	Improved sealing for flat strip caps*
PlateLoc Thermal Microplate Sealer with Small Hotplate and Peelable Aluminum Seal for PlateLoc Sealer	Please contact the SureSelect support team (see page 2) or your local representative for ordering information	Sealing wells for protocol steps performed inside or outside of the thermal cycler

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

2 Preparation and Fragmentation of Input DNA

This chapter 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 79.

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.

Step 1. Prepare and analyze quality of 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 25.

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 µl Buffer ATE in each round, for a final elution volume of approximately 60 µl.

NOTE If tissue lysis appears incomplete after one hour of digestion with Proteinase K, add an additional 10 µl 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 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 (Recommended Method)

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include a ΔΔCq DNA integrity score and the precise quantity of amplifiable DNA in the sample, allowing direct normalization of DNA input for each sample. DNA input recommendations based on ΔΔCq scores for individual samples are summarized in Table 9.

a 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.

b Remove a 1 µl aliquot of the FFPE gDNA sample for analysis using the Agilent NGS FFPE QC Kit to determine the ΔΔCq DNA integrity score. See the kit user manual at www.agilent.com for more information.

c For all samples with ΔΔCq DNA integrity score ≤1, use the Qubit-based gDNA concentration determined in step a, above, to determine volume of input DNA needed for the protocol.

d For all samples with ΔΔCq DNA integrity score >1, 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 9 SureSelect XT HS2 DNA input modifications based on ΔΔCq DNA integrity score

Protocol Parameter	non-FFPE Samples	FFPE Samples	
		ΔΔCq≤1*	ΔΔCq >1
DNA input for Library Preparation	10 ng to 200 ng DNA, based on Qubit Assay	10 ng to 200 ng DNA, based on Qubit Assay	10 ng to 200 ng of amplifiable DNA, based on qPCR quantification

* FFPE samples with ΔΔCq scores ≤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 Agilent's Genomic DNA ScreenTape assay DIN score

Agilent's Genomic DNA ScreenTape assay, used in conjunction with Agilent's TapeStation, 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 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.

b Remove a 1 µl aliquot of the FFPE gDNA sample and analyze using the Genomic DNA ScreenTape assay. See the user manual at www.agilent.com for more information.

c Using the DIN score reported for each sample in the Genomic DNA ScreenTape assay, consult Table 10 to determine the recommended amount of input DNA for the sample.

Table 10 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 using Covaris

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

The target fragment size and corresponding shearing conditions may vary for workflows using different NGS read lengths. See Table 11 for guidelines. Complete shearing instructions are provided on page 26.

Table 11 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 ×100 reads	150 to 200 bp	2 × 120 seconds	240 seconds
2 ×150 reads	180 to 250 bp	2 × 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.

NOTE

Shearing protocols have been optimized using a Covaris model E220 instrument and the 130-µl Covaris microTUBE. Consult the manufacturer's recommendations for use of other Covaris instruments or sample holders to achieve the desired target DNA fragment size.

1 Set up the Covaris E220 instrument. Refer to the instrument user guide.

- **a** Check that the water in the Covaris tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations.
- **b** Check that the water covers the visible glass part of the tube.
- **c** On the instrument control panel, push the Degas button. Degas the instrument according to the manufacturer's recommendations, typically 30–60 minutes.
- **d** Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C. Consult the manufacturer's recommendations for addition of coolant fluids to prevent freezing.

2 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 µl. 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.

3 Complete the DNA shearing steps below for each of the gDNA samples.

a Transfer the 50-µl DNA sample into a Covaris microTUBE, using a tapered pipette tip to slowly transfer the sample through the pre-split septum of the cap.

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 12.

Table 12 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	2 × 60 seconds	240 seconds
Bath Temperature	2° to 8° C	2° to 8° C	2° to 8° C

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

- Shear for 120 or 60 seconds (see Table 12)
- 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

e After completing the shearing step(s), put the Covaris microTUBE back into the loading and unloading station.

f While keeping the snap-cap on, insert a pipette tip through the pre-split septum, then slowly remove the sheared DNA.

g Transfer the sheared DNA sample (approximately 50 µl) to a 96-well plate or strip tube sample well. Keep the samples on ice.

h 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 g.

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 h.

The 50-µl sheared DNA samples are now ready for NGS sequencing library preparation, beginning with end repair/dA-tailing. Proceed to "Library Preparation" on page 31.

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 end-repair and dA-tailing.

Method 2: Enzymatic DNA Fragmentation

In this step, gDNA samples are fragmented using Agilent's SureSelect Enzymatic Fragmentation Kit for ILM (Pre PCR).

1 In wells of a thermal cycler-compatible strip tube or PCR plate, dilute 10 ng to 200 ng of each gDNA sample with nuclease-free water to a final volume of 7 µl.

2 Thaw the vial of 5X SureSelect Fragmentation Buffer, vortex, then place on ice.

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

Table 13 Thermal cycler program for enzymatic fragmentation*

Step	Temperature	Time
Step 1	37°C	Varies–see Table 14
Step 2	65°C	5 minutes
Step 3	4°C	Hold

* Use a reaction volume setting of 10 µl, if required for thermal cycler set up.

Optimal fragmentation conditions may vary based on the NGS read length to be used in the workflow. Refer to Table 14 below for the duration at 37°C appropriate for your sample type and required NGS read length.

Table 14 Fragmentation duration based on sample type and NGS read length

NGS read length requirement	Target fragment size	Duration of 37°C incubation step (Table 13)	
		High-quality DNA samples	FFPE DNA samples*
2 ×100 reads	150 to 200 bp	15 minutes	15 minutes
2 ×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 15.

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 15 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 µl	18 µl	50 µl
SureSelect Fragmentation Enzyme	1 µl	9 µl	25 µl
Total	3 µl	27 µl	75 µl

5 Add 3 µl of the Fragmentation master mix to each sample well containing 7 µl 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 plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 13.

8 When the program reaches the 4°C Hold step, remove the samples from the thermal cycler, add 40 µl of nuclease-free water to each sample, and place the samples on ice.

The 50-µl reactions are now ready for NGS sequencing library preparation, beginning with end repair/dA-tailing. Proceed to "Library Preparation" on page 31.

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 end-repair and dA-tailing.

3 Library Preparation

This chapter describes the steps to prepare DNA libraries for sequencing using the Illumina paired-read platform. For each sample to be sequenced, an individual dual-indexed and molecular-barcoded library is prepared. For an overview of the SureSelect XT HS2 NGS sample preparation workflow, see Figure 1 on page 10.

The NGS library preparation protocol that begins here is used for fragmented DNA samples produced by mechanical shearing (as detailed on page 25 to page 27) or produced by enzymatic fragmentation (as detailed on page 28 to page 29). Samples produced by either method should contain 10–200 ng of DNA fragments in a volume of 50 µl.

Protocol steps in this section use the components listed in Table 16. Thaw and mix each component as directed in Table 16 before use (refer to the *Where Used* column). Remove the AMPure XP beads from cold storage and equilibrate to room temperature for at least 30 minutes in preparation for use on page 37. *Do not freeze the beads at any time.*

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

Table 16 Reagents thawed before use in protocol

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
End Repair-A Tailing Buffer (yellow cap or bottle)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	page 35
Ligation Buffer (purple cap or bottle)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	page 33
End Repair-A Tailing Enzyme Mix (orange cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Place on ice just before use	Inversion	page 35
T4 DNA Ligase (blue cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Place on ice just before use	Inversion	page 33
SureSelect XT HS2 Adaptor Oligo Mix (clear cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	page 36

Step 1. Prepare the Ligation master mix

Prepare the Ligation master mix to allow equilibration to room temperature before use on page 36. Initiate this step before starting the End Repair/dA-tailing protocol; leave samples on ice while completing this step.

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

CAUTION

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

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

2 Prepare the appropriate volume of Ligation master mix by combining the reagents in Table 17.

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

Keep at room temperature for 30–45 minutes before use on page 36.

Table 17 Preparation of Ligation master mix

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

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

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

Step 2. Repair and dA-Tail the DNA ends

1 Preprogram a thermal cycler with the program in Table 18 for the End Repair and dA-Tailing steps. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 18 Thermal cycler program for End Repair/dA-Tailing*

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

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

NOTE When using Agilent's SureCycler 8800 thermal cycler, the heated lid may be left on (default setting) throughout the library preparation incubation steps. The heated lid must be on during the hybridization and amplification steps on page 40, page 51 and page 61.

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

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

3 Prepare the appropriate volume of End Repair/dA-Tailing master mix, by combining the reagents in Table 19.

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

Table 19 Preparation of End Repair/dA-Tailing master mix

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

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

5 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 18.

Step 3. Ligate the molecular-barcoded adaptor

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

2 Preprogram a thermal cycler with the program in Table 20 for the Ligation step. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 20 Thermal cycler program for Ligation*

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

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

3 To each end-repaired/dA-tailed DNA sample (approximately 70 µl), add 25 µl of the Ligation master mix that was prepared on page 33 and kept at room temperature. Mix by pipetting up and down at least 10 times using a pipette set to 70 µl or cap the wells and vortex at high speed for 5–10 seconds. Briefly spin the samples.

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

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

5 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 20.

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

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

Step 4. Purify the sample using AMPure XP beads

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

2 Prepare 400 µl of 70% ethanol per sample, plus excess, for use in step 8.

NOTE The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete Library Preparation protocol requires 0.8 ml of fresh 70% ethanol per sample.

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

4 Add 80 µl of homogeneous AMPure XP beads to each DNA sample (approximately 100 µl) in the PCR plate or strip tube. Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.

5 Incubate samples for 5 minutes at room temperature.

6 Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 to 10 minutes).

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

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

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

10 Repeat step 8 to step 9 once.

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

12 Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (typically 1–2 minutes).

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

13 Add 35 µl nuclease-free water to each sample well.

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

15 Incubate for 2 minutes at room temperature.

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

17 Remove the cleared supernatant (approximately 34 µl) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

NOTE It may not be possible to recover the entire 34-µl supernatant volume at this step; transfer the maximum possible amount of supernatant for further processing. To maximize recovery, transfer the cleared supernatant to a fresh well in two rounds of pipetting, using a P20 pipette set at 17 µl.

Step 5. Amplify the adaptor-ligated library

This step uses the components listed in Table 21. Before you begin, thaw the reagents listed below and keep on ice.

Table 21 Reagents for pre-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Pipette up and down 15–20 times	page 41
5× Herculase II Buffer with dNTPs (clear cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	page 41
SureSelect XT HS2 Index Primer Pairs	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR),* –20°C	Vortexing	page 41

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

1 Determine the appropriate index pair assignment for each sample. See Table 51 through Table 58 in the "Reference" chapter for 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.

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 in wells for subsequent experiments.

2 Preprogram a thermal cycler with the program in Table 22. Immediately pause the program, and keep paused until samples are loaded in step 6.

Table 22 Pre-Capture PCR Thermal Cycler Program*

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	See Table 23 for cycle number	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

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

Table 23 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 24, on ice. Mix well on a vortex mixer.

Table 24 Preparation of Pre-Capture PCR Reaction Mix

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

4 Add 11 µl of the PCR reaction mixture prepared in Table 24 to each purified DNA library sample (34 µl) in the PCR plate wells.

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

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

6 Before adding the samples to the thermal cycler, resume the thermal cycling program in Table 22 to bring the temperature of the thermal block to 98°C. Once the cycler has reached 98°C, immediately place the sample plate or strip tube in the thermal block and close the lid.

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

Step 6. Purify the amplified library with AMPure XP beads

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

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

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

Step 7. Assess quality and quantity

Analyze a five-fold dilution of each sample using one of the platforms listed in Table 25. Follow the instructions in the linked user guide provided for each assay in Table 25, after reviewing the SureSelect library qualification steps on page 45. Each analysis method provides an electropherogram showing the size distribution of fragments in the sample and tools for determining the concentration of DNA in the sample. See Table 26 for fragment size distribution guidelines for various sample types. Representative electropherograms generated using the TapeStation system are provided to illustrate typical results for libraries prepared from several types of input DNA.

Table 25 Pre-capture library analysis options

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

Table 26 Pre-capture library qualification guidelines

NGS read length for fragmentation protocol selection	Fragmentation method	Input DNA type	Expected library DNA fragment size peak position
2 ×100 reads	Mechanical shearing	Intact DNA	300 to 400 bp
		FFPE DNA	200 to 400 bp
	Enzymatic fragmentation	Intact DNA	300 to 400 bp
		FFPE DNA	200 to 400 bp
2 ×150 reads	Mechanical shearing	Intact DNA	330 to 450 bp
		FFPE DNA	200 to 450 bp
	Enzymatic fragmentation	Intact DNA	330 to 450 bp
		FFPE DNA	200 to 450 bp

1 Set up the instrument as instructed in the appropriate user guide (links provided in Table 25).

2 Prepare samples for analysis by diluting 1 µl of each prepared library sample in 4 µl of nuclease-free water.

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

4 Verify that the electropherogram shows the expected DNA fragment size peak position (see Table 26 for guidelines). Sample TapeStation system electropherograms are shown for libraries prepared from sheared DNA designed for 2 ×100 bp reads in Figure 2 (high-quality DNA), Figure 3 (medium-quality FFPE DNA), and Figure 4 (low-quality FFPE DNA).

   Electropherograms obtained using the other analysis platform options listed in Table 25 are expected to show similar fragment size profiles.

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

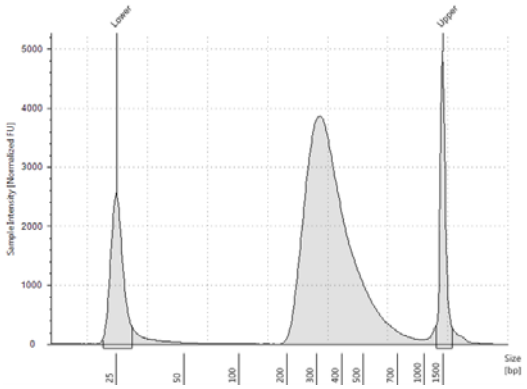


Figure 2 Pre-capture library prepared from a sheared high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

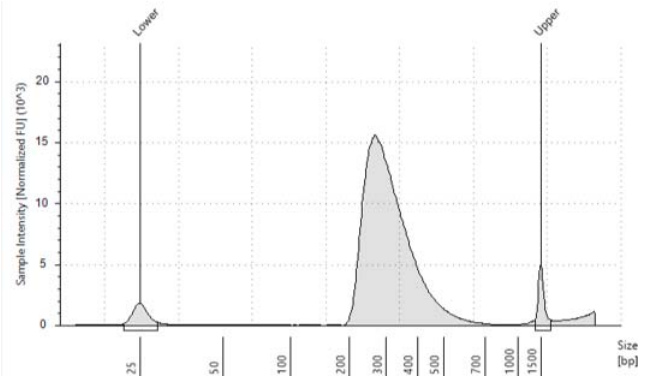


Figure 3 Pre-capture library prepared from a typical FFPE gDNA sample (fragmented by shearing) analyzed using a D1000 ScreenTape assay.

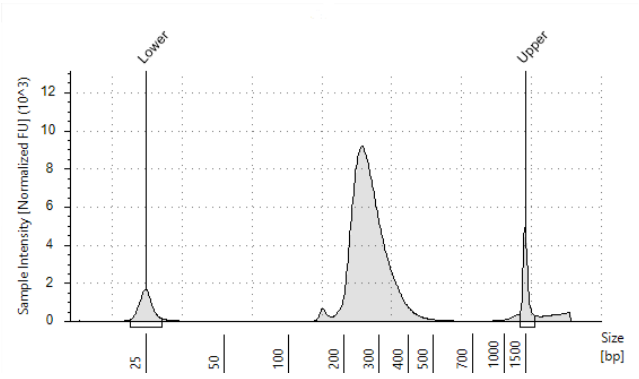


Figure 4 Pre-capture library prepared from a low-quality FFPE gDNA sample (fragmented by shearing) analyzed using a D1000 ScreenTape assay.

Observation of a low molecular weight peak, in addition to the expected library fragment peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to that seen in the example electropherogram in Figure 4. See Troubleshooting on page 100 for additional considerations.

NOTE

For libraries being prepared for whole-genome sequencing (not specifically supported by this user guide), 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 42.

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 and Capture

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

The standard single-day SureSelect XT HS2 protocol includes the hybridization step immediately followed by capture and amplification steps. If required, the hybridized samples may be held overnight with capture and amplification steps completed the following day by using the simple protocol modifications noted on page 52.

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

Step 1. Hybridize DNA libraries to the probe

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

The hybridization reaction requires 500-1000 ng of prepared DNA in a volume of 12 µl. Use the maximum amount of prepared DNA available in this range.

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

Table 27 Reagents for Hybridization

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect XT HS2 Blocker Mix (blue cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	page 52
SureSelect RNase Block (purple cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	page 53
SureSelect Fast Hybridization Buffer (bottle)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw and keep at Room Temperature	page 53
Probe	–80°C	Thaw on ice	page 53

1 Preprogram a thermal cycler (with heated lid ON) with the program in Table 28 for the SureSelect XT HS Human All Exon V8 Probe or in Table 29 for all other probes. Immediately pause the program, and keep paused until samples are loaded in step 4 on page 52.

Table 28 Hybridization program **for SureSelect XT HS Human All Exon V8 Probe** *

Segment #	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	65°C	10 minutes
3	1	65°C	1 minute (Pause cycler here for reagent addition; see step 7 on page 54)
4	60	65°C	1 minute
		37°C	3 seconds
5	1	65°C	60 minutes
6	1	65°C	Hold briefly until ready to begin capture steps on page 56

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

Table 29 Hybridization program **for all other probes** *

Segment #	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	65°C	10 minutes
3	1	65°C	1 minute (Pause cycler here for reagent addition; see step 7 on page 54)
4	60	65°C†	1 minute
		37°C	3 seconds
5	1	65°C†	Hold briefly until ready to begin capture steps on page 56

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

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

NOTE

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

- In the final segment of the thermal cycler program (Table 28 or Table 29), replace the 65°C Hold step with a 21°C Hold step.
- The hybridized samples may be held at 21°C for up to 16 hours. Begin the capture preparation steps on page 55 on day 2, after the overnight hold.

2 Place 1000 ng of each prepared gDNA library sample into the hybridization plate or strip tube wells and then bring the final volume in each well to 12 µl using nuclease-free water. If 1000 ng DNA is not available for any of the samples, use the maximum amount available, within the 500–1000 ng range.

3 To each DNA library sample well, add 5 µl of SureSelect XT HS2 Blocker Mix (blue cap). Seal the wells then vortex at high speed for 5 seconds. Spin briefly to collect the liquid and release any bubbles.

CAUTION

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

4 Transfer the sealed sample plate or strip to the thermal cycler and resume the thermal cycling program (Table 28 or Table 29 on page 51), allowing the cycler to complete Segments 1 and 2 of the program.

Important: The thermal cycler must be paused during Segment 3 to allow additional reagents to be added to the Hybridization wells in step 7 on page 54.

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

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

Table 30 Preparation of RNase Block solution

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

NOTE

Prepare the mixture described in step 6, below, just before pausing the thermal cycler in Segment 3. Keep the mixture at room temperature briefly until the mixture is added to the DNA samples in step 7 on page 54. Do not keep solutions containing the probe at room temperature for extended periods.

6 Prepare the Probe Hybridization Mix appropriate for your probe design size. Use Table 31 for probes ≥3 Mb or Table 32 for probes <3 Mb.

Combine the listed reagents at room temperature. Mix well by vortexing at high speed for 5 seconds then spin down briefly. Proceed immediately to step 7.

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

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
25% RNase Block solution (from step 5)	2 µl	18 µl	50 µl
Probe (with design ≥3 Mb)	5 µl	45 µl	125 µl
SureSelect Fast Hybridization Buffer	6 µl	54 µl	150 µl
Total	**13 µl**	**117 µl**	**325 µl**

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

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

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

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

The hybridization reaction wells now contain approximately 30 µl.

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

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

CAUTION

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

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

Step 2. Prepare streptavidin-coated magnetic beads

The remaining hybridization capture steps use the reagents in Table 33.

NOTE

If performing same-day hybridization and capture, begin the bead preparation steps below approximately one hour after starting hybridization in step 9 on page 54. If performing next-day capture after an overnight hold at 21°C, begin the bead preparation steps below on day 2, just before you are ready to start the capture steps on page 56.

Table 33 Reagents for Capture

Kit Component	Storage Location	Where Used
SureSelect Binding Buffer	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	page 55
SureSelect Wash Buffer 1	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	page 56
SureSelect Wash Buffer 2	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	page 56
SureSelect Streptavidin Beads **OR** Dynabeads MyOne Streptavidin T1 Beads	4°C	page 55

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

NOTE

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

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

1. After all streptavidin bead preparation steps are complete and with the hybridization thermal cycling program in the final hold segment (see Table 28 or Table 29 on page 51), transfer the samples to room temperature.
2. Immediately transfer the entire volume (approximately 30 µl) of each hybridization mixture to wells containing 200 µl of washed streptavidin beads using a multichannel pipette.

   Pipette up and down 5–8 times to mix then seal the wells with fresh caps.
3. Incubate the capture plate or strip tube on a 96-well plate mixer, mixing vigorously (at 1400–1900 rpm), for 30 minutes at room temperature.

   Make sure the samples are properly mixing in the wells.
4. During the 30-minute incubation for capture, prewarm SureSelect Wash Buffer 2 at 70°C as described below.
   a. Place 200-µl aliquots of Wash Buffer 2 in wells of a fresh 96-well plate or strip tubes. Aliquot 6 wells of buffer for each 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, then remove and discard the supernatant.
7. Resuspend the beads in 200 µl of SureSelect Wash Buffer 1. Mix by pipetting up and down 15–20 times, until beads are fully resuspended.
8. Put the plate or strip tube in the magnetic separator. Wait for the solution to clear (approximately 1 minute), then remove and discard 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 protocol steps below.

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

b 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 µl of nuclease-free water to each sample well. Pipette up and down 8 times to resuspend the beads.

Keep the samples on ice until they are used on page 62.

NOTE

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

This chapter describes the steps to amplify, purify, and assess quality and quantity of the captured libraries. Sample pooling instructions are provided to prepare the indexed, molecular barcoded samples for multiplexed sequencing. Sequencing run setup and sequencing data analysis steps will vary according to your NGS platform and data analysis pipeline; guidelines for these downstream steps are also provided in this chapter.

Step 1. Amplify the captured libraries

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

This step uses the components listed in Table 34. Before you begin, thaw the reagents listed below and keep on ice. Remove the AMPure XP beads from cold storage and equilibrate to room temperature for at least 30 minutes in preparation for use on page 63. *Do not freeze the beads at any time.*

Table 34 Reagents for post-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Pipette up and down 15–20 times	page 62
5× Herculase II Buffer with dNTPs (clear cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Vortexing	page 62
SureSelect Post-Capture Primer Mix (clear cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Vortexing	page 62

Prepare one amplification reaction for each DNA library.

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

1 Preprogram a thermal cycler with the program in Table 35. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 35 Post-capture PCR Thermal Cycler Program*

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10–16 (See Table 36 for hybridization probe design size-based cycle number recommendations)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

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

Table 36 Post-capture PCR cycle number recommendations

Probe Capture Library 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 probes)	10–11 cycles

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 37, on ice. Mix well on a vortex mixer.

Table 37 Preparation of post-capture PCR reaction mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
Nuclease-free water	13 µl	117 µl	325 µl
5× Herculase II Buffer with dNTPs (clear cap)	10 µl	90 µl	250 µl
Herculase II Fusion DNA Polymerase (red cap)	1 µl	9 µl	25 µl
SureSelect Post-Capture Primer Mix (clear cap)	1 µl	9 µl	25 µl
Total	**25 µl**	**225 µl**	**625 µl**

3 Add 25 µl of the PCR reaction mix prepared in Table 37 to each sample well containing 25 µl of bead-bound target-enriched DNA (prepared on page 57 and held on ice).

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

5 Place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 35.

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

The beads can be discarded at this time.

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

1. Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use.
2. Prepare 400 µl of fresh 70% ethanol per sample, plus excess, for use in step 8.
3. Mix the AMPure XP bead suspension well so that the suspension appears homogeneous and consistent in color.
4. Add 50 µl of the homogeneous AMPure XP bead suspension to each amplified DNA sample (approximately 50 µl) in the PCR plate or strip tube. Mix well by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds.

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 3 to 5 minutes).
7. Keep the plate or tubes in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not disturb the beads while removing the solution.
8. Continue to keep the plate or tubes in the magnetic stand while you dispense 200 µl of freshly-prepared 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 and step 9 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.
11. Seal the wells with strip caps, then briefly spin to collect the residual ethanol. Return the plate or strip tube to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
12. Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (typically 1–2 minutes).
13. Add 25 µl of Low TE to each sample well.

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

15 Incubate for 2 minutes at room temperature.

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

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

Step 3. Assess sequencing library DNA quantity and quality

Analyze each library using one of the platforms listed in Table 38. Follow the instructions in the linked user guide provided for each assay in Table 38, after reviewing the post-capture library qualification steps on page 66. See Table 39 for fragment size distribution guidelines for various sample types. Representative electropherograms generated using the TapeStation system are provided to illustrate typical results for post-capture libraries prepared from selected sample types.

Table 38 Post-capture library analysis options

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

Table 39 Post-capture library qualification guidelines

NGS read length for fragmentation protocol selection	Input DNA type	Expected DNA fragment size peak position
2 ×100 reads	Intact DNA	200 to 400 bp (see Figure 5 for sample electropherogram)
	FFPE DNA	200 to 400 bp (see Figure 6 and Figure 7 for sample electropherograms)
2 ×150 reads	Intact DNA	230 to 450 bp
	FFPE DNA	200 to 450 bp

1 Set up the instrument as instructed in the appropriate user guide (links provided in Table 38).

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

3 Verify that the electropherogram shows the expected DNA fragment size peak position (see Table 39 for guidelines). Sample TapeStation system electropherograms are shown for libraries prepared from sheared DNA designed for 2 ×100 bp reads in Figure 5 (high-quality input DNA), Figure 6 (medium-quality FFPE input DNA), and Figure 7 (low-quality FFPE input DNA).

   Electropherograms obtained using the other analysis platform options listed in Table 38 are expected to show similar fragment size profiles.

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

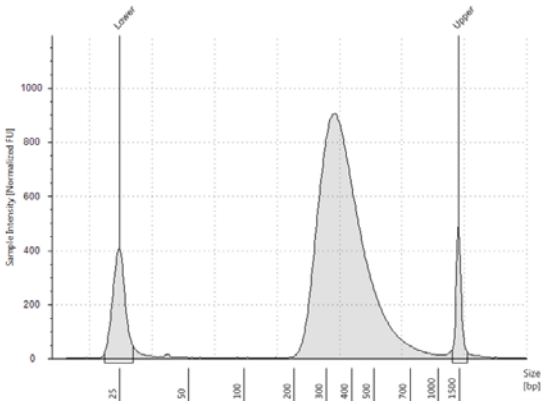


Figure 5 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

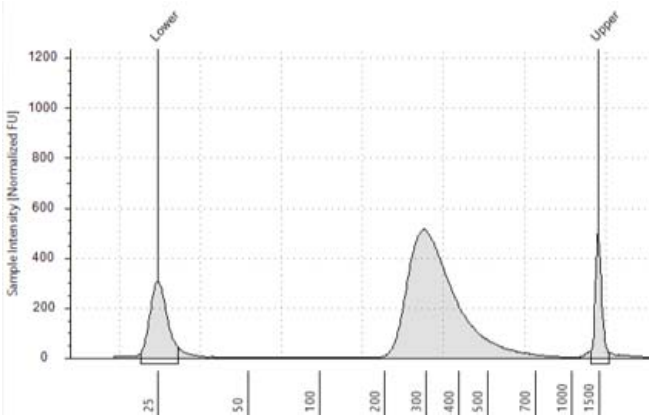


Figure 6 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

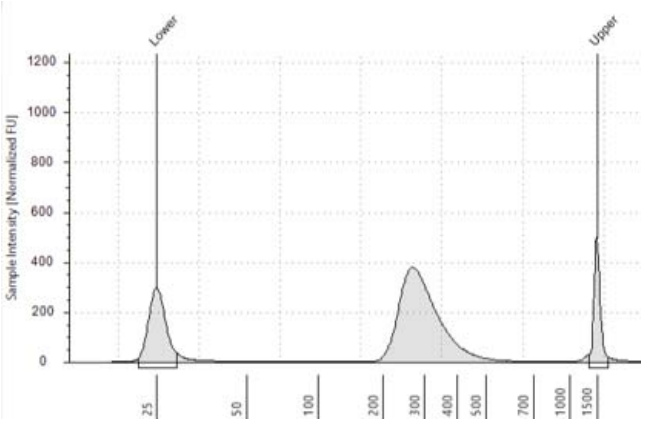


Figure 7 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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

Step 4. Pool samples for multiplexed sequencing

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

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

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

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

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

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

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

is the number of indexes, and

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

Table 40 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µl at 10 nM DNA.

Table 40 Example of volume calculation for total volume of 20 µl at 10 nM concentration

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

If you store the library before sequencing, add Tween 20 to 0.1% v/v and store at -20°C short term.

Step 5. Prepare sequencing samples

The final SureSelect XT HS2 library pool is ready for direct sequencing using standard Illumina paired-end primers and chemistry. Each fragment in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform, as shown in Figure 8.

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

Libraries can be sequenced on the Illumina HiSeq, MiSeq, NextSeq or NovaSeq platform using the run type and chemistry combinations shown in Table 41.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See Table 41 for kit configurations compatible with the recommended read length.

The optimal seeding concentration for SureSelect XT HS2 target-enriched libraries varies according to sequencing platform, run type, and Illumina kit version. See Table 41 for guidelines. 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 41.

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

Table 41 Illumina Kit Configuration Selection Guidelines

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

Step 6. Do the sequencing run and analyze the data

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

- Each of the sample-level indexes requires an 8-bp index read. For complete index sequence information, see Table 51 on page 87 through Table 58 on page 94.
- For the HiSeq, NextSeq, and NovaSeq platforms, set up the run using the instrument's user interface, following the guidelines on page 73.
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed on page 73 to page 76 to generate a custom sample sheet.
- Demultiplex using Illumina's bcl2fastq software to generate paired end reads based on the dual indexes and remove sequences with incorrectly paired P5 and P7 indexes.
- The MBC sequence and dark bases are located at the 5' end of both Read 1 and Read 2. Use the Agilent Genomics NextGen Toolkit (AGeNT) for molecular barcode extraction and trimming (see page 77 for more information). If your sequence analysis pipeline excludes MBCs and is incompatible with AGeNT, you can trim or mask the first five bases from each read before alignment as described in the *Note* on page 77.
- Before aligning reads to the reference genome, Illumina adaptor sequences should be trimmed from the reads using Agilent's AGeNT trimmer module, which properly accounts for the degenerate MBCs in the adaptor sequence. See page 77 for more information. Do not use the adaptor trimming options in Illumina Experiment Manager (IEM). Make sure any IEM adaptor trimming option checkboxes are cleared (deselected) when setting up the sequencing run.

HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface, using the settings shown in Table 42. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface and enter the **Cycles** settings in Table 42.

For the NextSeq or NovaSeq platform, open the *Run Setup* screen of the instrument control software interface and enter the **Read Length** settings in Table 42. In the **Custom Primers** section, clear (do **not** select) the checkboxes for all primers (*Read 1, Read 2, Index 1* and *Index 2*).

Table 42 Run settings

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

MiSeq platform sequencing run setup guidelines

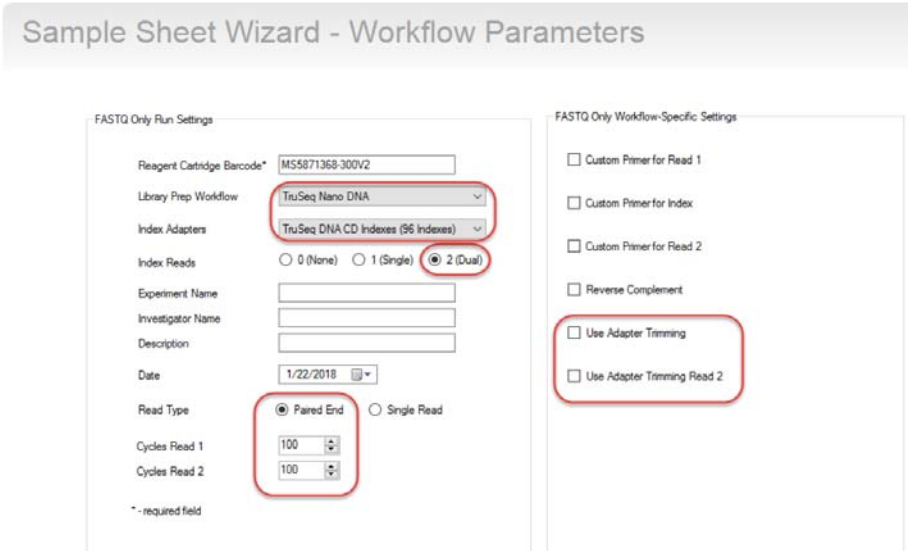
Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below. Once a Sample Sheet has been generated, index sequences need to be manually changed to the SureSelect XT HS2 indexes used for each sample. See page 87 through page 94 for nucleotide sequences of the SureSelect XT HS2 index pairs.

Set up a custom Sample Sheet:

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

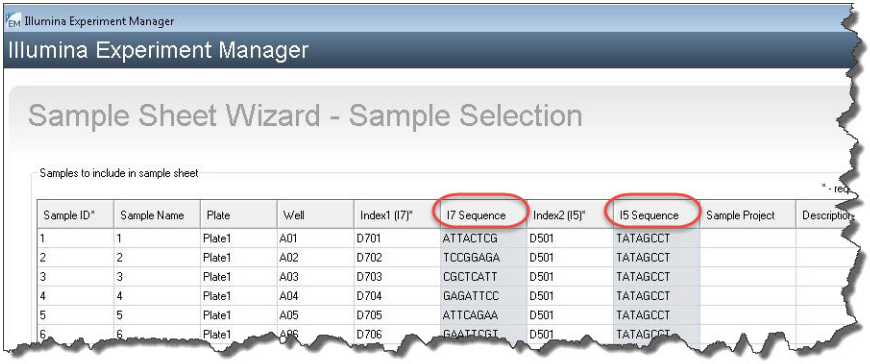
2 On the **Workflow Parameters** screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA**. In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. Clear both adaptor-trimming checkboxes under *FASTQ Only Workflow-Specific Settings*, since adaptor trimming must be performed using Agilent's AGeNT software (see page 77).

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



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

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



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

Editing the Sample Sheet to include SureSelect XT HS2 dual indexes

- Open the Sample Sheet file in a text editor and edit the i7 and i5 index information for each sample in columns 5–8 (highlighted below). See page 87 through page 94 for nucleotide sequences of the SureSelect XT HS2 indexes.
- In column 5 under **I7_Index_ID**, enter the SureSelect XT HS2 index pair number assigned to the sample. In column 6 under **index**, enter the corresponding P7 index sequence.
- In column 7 under **I5_Index_ID**, enter the SureSelect XT HS2 index pair number assigned to the sample. In column 8 under **index2**, enter the corresponding P5 index sequence.
- If the run includes more than 96 samples, the sample sheet may be edited to include additional sample rows containing the assigned SureSelect XT HS2 index pair sequences in column 6 (P7 index) and column 8 (P5 index).

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

Figure 9 Sample sheet for SureSelect XT HS2 library sequencing

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

Sequence analysis resources

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

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

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

NOTE

If your sequence analysis pipeline excludes MBCs, you can remove the first 5 bases from Read 1 and Read 2 by either masking or trimming before proceeding to further analysis. To remove during demultiplexing via masking, include the base mask **N5Y*,I8,I8,N5Y*** (where * may be replaced with the actual read length, matching the read length value in the RunInfo.xml file). Alternatively, the first 5 bases may be trimmed from the demultiplexed fastq files using a suitable processing tool of your choice, such as seqtk. Alternatively, the AGeNT trimmer module can be used to remove the MBCs and properly remove adaptor sequences as well. Standard adaptor trimmers will fail to remove the MBC sequences from the opposite adaptor (refer to Figure 8).

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

6 Appendix: Using FFPE-derived DNA Samples

This chapter summarizes 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 43.

Table 43 Summary of protocol modifications for FFPE samples

Workflow Step and page	Parameter	Condition for non-FFPE Samples	Condition for FFPE Samples
gDNA Sample Preparation page 22	Qualification of DNA Integrity	Not required	Required
DNA input for Library Preparation page 22	Input amount and means of quantification	10 ng to 200 ng, quantified by Qubit assay	Based on determined DNA integrity (see Table 9 on page 23 and Table 10 on page 24)
DNA Shearing page 26	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 page 40	Cycle number	8–11	11–14
Sequencing page 81	Output augmentation	Per project requirements	1× to 10× based on determined DNA integrity (see Table 44 and Table 45 on page 81)

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 ΔΔCq 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 ΔΔCq: For samples qualified based on the ΔΔCq DNA integrity score, use the guidelines in Table 44. For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with ΔΔCq score of 1 requires 200–400 Mb of sequencing output to achieve the same coverage.

Table 44 Recommended sequencing augmentation for FFPE-derived DNA samples

ΔΔCq value	Recommended fold increase for FFPE-derived sample
<0.5	No extra sequencing output
between 0.5 and 2	Increase sequencing allocation by 2× to 4×
>2	Increase sequencing allocation by 5× to 10× or more

Samples qualified using DIN: For samples qualified based on the Genomic DNA ScreenTape assay DIN integrity score, use the guidelines in Table 45. 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 45 Recommended sequencing augmentation for FFPE-derived DNA samples

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

7 Reference

This chapter contains reference information, including component kit contents, index sequences, troubleshooting information, and a quick-reference protocol for experienced users.

Kit Contents

SureSelect XT HS2 Target Enrichment System Reagent Kits include the component kits listed in Table 46. Detailed contents of each of the multi-part component kits listed in Table 46 are shown in Table 47 through Table 50 on the following pages.

Table 46 Component Kits

Component Kit Name	Storage Condition	Component Kit Part Number	
		16 Reaction Kits	96 Reaction Kits
Standard Component Modules			
SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0146	5500-0147
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	–20°C	5191-5687 (Index Pairs 1–16)	5191-5688 (Index Pairs 1–96), 5191-5689 (Index Pairs 97–192), 5191-5690 (Index Pairs 193–288), OR 5191-5691 (Index Pairs 289–384)
SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	Room Temperature	5190-9685	5190-9687
SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	–20°C	5191-6686	5191-6688
Optional Component Modules			
SureSelect DNA AMPure® XP Beads	+4°C	5191-5739*	5191-5740†
SureSelect Streptavidin Beads	+4°C	5191-5741*	5191-5742†
SureSelect Enzymatic Fragmentation Kit	–20°C	5191-4079*	5191-4080‡

* Provided with the 16-Reaction SureSelect XT HS2 DNA Starter Kit, p/n G9982A.

† Provided with 96-Reaction Reagent Kit part numbers G9984A, G9984B, G9984C, G9984D.

‡ Purchased separately; not included with SureSelect XT HS2 DNA Reagent Kits, but use is supported by the protocols in this publication.

Table 47 SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR) Content

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

Table 48 SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) Content

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

Table 49 SureSelect Target Enrichment Kit, ILM Hyb Module Box 1 (Post PCR) Content

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

Table 50 SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR) Content

Kit Component	16 Reaction Kit Format	96 Reaction Kit Format
SureSelect Fast Hybridization Buffer	bottle	bottle
SureSelect XT HS2 Blocker Mix	tube with blue cap	tube with blue cap
SureSelect RNase Block	tube with purple cap	tube with purple cap
SureSelect Post-Capture Primer Mix	tube with clear cap	tube with clear cap
Herculase II Fusion DNA Polymerase	tube with red cap	tube with red cap
5× Herculase II Reaction Buffer with dNTPs	tube with clear cap	tube with clear cap

SureSelect XT HS2 Index Primer Pair Information

The SureSelect XT HS2 Index Primer Pairs are provided pre-combined. Each member of the primer pair contains a unique 8-bp P5 or P7 index, resulting in dual-indexed NGS libraries. The nucleotide sequence of the index portion of each primer is provided in Table 51 through Table 58. See page 72 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

NOTE

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

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

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

One primer pair is provided in each well of 8-well strip tubes (16 reaction kits; see Figure 10 for a map) or of 96-well plates (96 reaction kits; see page 96 through page 97 for plate maps). Each well contains a single-use aliquot of a specific pair of forward plus reverse primers.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Table 58 SureSelect XT HS2 Index Primer Pairs 337–384, provided in red 96-well plate

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

Index Primer Pair Strip Tube and Plate Maps

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

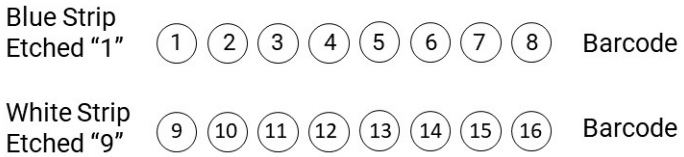


Figure 10 Map of the SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) strip tubes provided with 16 reaction kits

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

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

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

See Table 59 on page 96 through Table 62 on page 97 for plate maps showing positions of the SureSelect XT HS2 Index Primer Pairs provided with 96 reaction kits.

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

Table 59 Plate map for SureSelect XT HS2 Index Primer Pairs 1-96, provided in orange plate

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

Table 60 Plate map for SureSelect XT HS2 Index Primer Pairs 97-192, provided in blue plate

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

Table 61 Plate map for SureSelect XT HS2 Index Primer Pairs 193-288, provided in green plate

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

Table 62 Plate map for SureSelect XT HS2 Index Primer Pairs 289-384, provided in red plate

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

Troubleshooting Guide

If 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 yield of pre-capture libraries is low

- ✔ The library preparation protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance of the highly viscous buffer and enzyme solutions used in the protocol. Be sure to adhere to all instructions when setting up the reactions.
- ✔ Ensure that the ligation master mix (see page 33) is kept at room temperature for 30–45 minutes before use.
- ✔ PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the pre-capture PCR cycle number by 1 to 2 cycles. 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 degraded samples, including 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.

- ✔ Performance of the solid-phase reversible immobilization (SPRI) purification step may be poor. Verify the expiration date for the vial of AMPure XP beads used for purification. Adhere to all bead storage and handling conditions recommended by the manufacturer. Ensure that the beads are kept at room temperature for at least 30 minutes before use. Use freshly-prepared 70% ethanol for each SPRI procedure.
- ✔ DNA elution during SPRI purification steps may be incomplete. Ensure that the AMPure XP beads are not overdried just prior to sample elution.

If solids observed in the End Repair-A Tailing Buffer

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

If pre-capture library fragment size is 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 input DNA that are smaller than the target DNA shear size.
- ✔ DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color and verify that you are using the bead volume recommended for pre-capture purification on page 42.

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

- ✔ The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to the example shown in Figure 4 on page 46. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield of pre-capture libraries. If excessive adaptor-dimers are observed, verify that the adaptor ligation protocol is being performed as directed on page 36. In particular, ensure that the Ligation master mix is mixed with the sample prior to adding the 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 42.

If yield of post-capture libraries is low

- ✔ PCR cycle number may require optimization. Repeat library preparation and target enrichment for the sample, increasing the post-capture PCR cycle number by 1 to 2 cycles.
- ✔ The RNA Probe used for hybridization may have been compromised. Verify the expiration date on the Probe vial or Certificate of Analysis. Adhere to the recommended storage and handling conditions. Ensure that the Capture Library Hybridization Mix is prepared immediately before use, as directed on page 53, and that solutions containing the Probe are not held at room temperature for extended periods.

If post-capture library fragment size is different than expected in electropherograms

- ✔ DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color and verify that you are using the bead volume recommended for post-capture purification on page 63.

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

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

- SureSelect Wash Buffer 2 is pre-warmed to 70°C (see page 56)
- Samples are maintained at 70°C during washes (see page 57)
- Bead suspensions are mixed thoroughly during washes by pipetting up and down **and** vortexing (see page 57)

✔ Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a vortex and plate spinner or centrifuge in close proximity to thermal cycler to retain the 65°C sample temperature during mixing and transfer steps (step 8 to step 9 on page 54).

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

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

- For libraries target-enriched using the SureSelect XT HS Human All Exon V8 Probe and the hybridization program in Table 28 on page 51 (including segment with one-hour incubation at 65°C), repeat target enrichment using the hybridization program in Table 29 on page 51 (without the one-hour incubation at 65°C segment).
- For other probes, repeat target enrichment at lower stringency using a modified thermal cycler program for hybridization, reducing the hybridization temperature in segments 4 and 5 from 65°C to 62.5°C or 60°C (see Table 29 on page 51).

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

Quick Reference Protocol

An abbreviated summary of the protocol steps is provided below for experienced users. Use the complete protocol on page 22 to page 65 until you are familiar with all of the protocol details such as reagent mixing instructions and instrument settings.

Step	Summary of Conditions
	Library Prep
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 page 23 and page 24 Mechanically shear DNA using Covaris with shearing conditions on page 25 OR enzymatically fragment DNA using SureSelect Enzymatic Fragmentation Kit with protocol on page 28 (50 µl final volume)
Prepare Ligation master mix	Per reaction: 23 µl Ligation Buffer + 2 µl T4 DNA Ligase Keep at room temperature 30–45 min before use
Prepare End-Repair/dA-Tailing master mix	Per reaction: 16 µl End Repair-A Tailing Buffer + 4 µl End Repair-A Tailing Enzyme Mix Keep on ice
End-Repair and dA-Tail the DNA fragments	50 µl 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 Incubate in thermal cycler: 30 min @ 20°C, Hold @ 4°C
Purify DNA	100 µl DNA sample + 80 µl AMPure XP bead suspension Elute DNA in 35 µl nuclease-free H_2O, removing 34 µl to fresh well
Prepare PCR master mix	Per reaction: 10 µl 5× Herculase II Reaction Buffer with dNTPs + 1 µl Herculase II Fusion DNA Polymerase Keep on ice
Amplify the purified DNA	34 µl purified DNA + 11 µl PCR master mix + 5 µl assigned SureSelect XT HS2 Index Primer Pair Amplify in thermal cycler using program on page 40
Purify amplified DNA	50 µl amplified DNA + 50 µl AMPure XP bead suspension Elute DNA in 15 µl nuclease-free H_2O
Quantify and qualify DNA	Analyze quantity and quality using TapeStation, Bioanalyzer, or Fragment Analyzer System

Step	Summary of Conditions
	Hybridization/Capture
Program thermal cycler	Input the appropriate thermal cycler program on page 51 and pause program
Prep DNA in hyb plate	Adjust 500–1000 ng purified prepared library to 12 µl volume with nuclease-free H_2O
Run pre-hybridization blocking protocol	12 µl library DNA + 5 µl SureSelect XT HS2 Blocker Mix Run paused thermal cycler program segments 1 through 3; start new pause during segment 3 (1 min @ 65°C)
Prepare Hyb Mix	Prepare 25% RNAse Block dilution, then prepare appropriate Capture Library Hyb Mix below: **Probes ≥3 Mb:** 2 µl 25% RNase Block + 5 µl Probe+ 6 µl SureSelect Fast Hybridization Buffer **Probes <3 Mb:** 2 µl 25% RNase Block + 2 µl Probe + 3 µl nuclease-free H_2O + 6 µl SureSelect Fast Hybridization Buffer
Run the hybridization	With cycler paused and samples retained in cycler, add 13 µl Capture Library Hyb Mix to wells Resume the thermal cycler program, completing the remaining hybridization segment(s) and 65°C or 21°C hold segment
Prepare streptavidin beads	Wash 50 µl Streptavidin T1 beads 3× in 200 µl SureSelect Binding Buffer
Capture hybridized libraries	Add hybridized samples (~30 µl) to washed streptavidin beads (200 µl) Incubate 30 min at RT with vigorous shaking (1400-1900 rpm) During incubation, pre-warm 6 × 200 µl aliquots per sample of SureSelect Wash Buffer 2 to 70°C
Wash captured libraries	Collect streptavidin beads with magnetic stand, discard supernatant Wash beads 1× with 200 µl SureSelect Wash Buffer 1 at RT Wash beads 6× with 200 µl pre-warmed SureSelect Wash Buffer 2 (5 minutes at 70°C per wash) Resuspend washed beads in 25 µl nuclease-free H_2O
	Post-capture amplification
Prepare PCR master mix	Per reaction: 13 µl nuclease-free H_2O+ 10 µl 5× Herculase II Reaction Buffer with dNTPs + 1 µl SureSelect Post-Capture Primer Mix + 1 µl Herculase II Fusion DNA Polymerase Keep on ice
Amplify the bead-bound captured libraries	25 µl DNA bead suspension+ 25 µl PCR master mix Amplify in thermal cycler using conditions on page 61
Purify amplified DNA	Remove streptavidin beads using magnetic stand; retain supernatant 50 µl amplified DNA + 50 µl AMPure XP bead suspension Elute DNA in 25 µl Low TE
Quantify and qualify DNA	Analyze quantity and quality using TapeStation, Bioanalyzer, or Fragment Analyzer System

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In This Book

This guide contains instructions for using the SureSelect XT HS2 DNA Reagent Kits and SureSelect probes to prepare target-enriched NGS libraries for the Illumina platform.

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