SureSelect XT HS2 DNA Kits

Library Preparation (+/-MBC) / Overnight-Hyb Target Enrichment / Post-capture Pooling Workflow

For Illumina Platform NGS

Protocol

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SureSelect platform manufactured with Agilent SurePrint technology.

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Notices

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In this Guide...

This guide provides an optimized protocol for preparation of target-enriched Illumina paired-end multiplexed sequencing libraries using SureSelect XT HS2 DNA Reagent Kits. The specific workflow supported by this guide includes Library Preparation (+/- dual molecular barcodes or MBCs) with Overnight-Hyb Target Enrichment and Post-capture library pooling.

1 Before You Begin

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

2 Input DNA Preparation and Fragmentation

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

3 Library Preparation using +/- MBC Adaptors

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

4 Hybridization, Capture, and Amplification

This section describes the steps to hybridize and capture targeted fragments in the prepared DNA library using a SureSelect Probe, using overnight hybridization conditions and the corresponding reagents. The post-capture library amplification through final library QC steps of the workflow are also included in this section.

5 NGS and Analysis Guidelines

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

6 Appendix 1: Using FFPE-derived DNA Samples

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

7 Appendix 2: Protocol Modifications for RNA Libraries

This section describes the protocol modifications for RNA libraries prepared from cDNA fragments.

8 Reference

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

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1 Before You Begin

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

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

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 supported by this publication is summarized in Figure 1. The estimated time requirements for each step are summarized in Table 1. The protocols provided in this publication may also be used for RNA libraries using the modifications detailed in "Appendix 2: Protocol Modifications for RNA Libraries" on page 56.

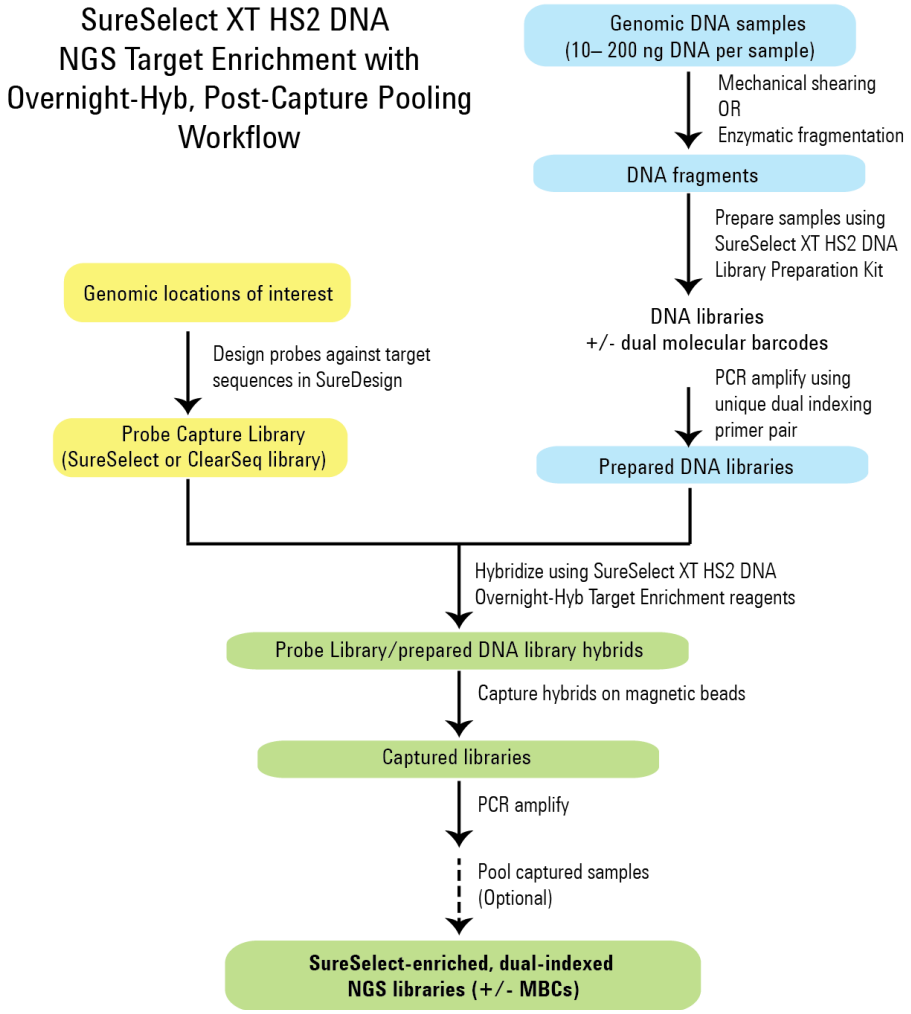

Figure 1 Overall target-enriched sequencing sample preparation workflow.

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

Step	Time
DNA fragmentation, library preparation and clean-up	2.25 hours
Pre-capture amplification and clean-up, QC using TapeStation	1.5 hour
Hybridization and Capture	18 hours (overnight)
Post-capture amplification and clean-up, QC using TapeStation	1.5 hours

* Estimates are provided as guidelines for 16 reaction runs using 200 ng high-quality input DNA, enzymatic DNA fragmentation, and probe design >5 Mb. Your results may vary.

Procedural and Safety Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Use best-practices to prevent PCR product and nuclease contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow.
  2. Maintain clean work areas. Clean the surfaces that pose the highest risk of contamination daily using a 10% bleach solution, or equivalent.
  3. Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
  4. Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.
- Several reagent solutions used in the SureSelect XT HS2 protocols are highly viscous. Make sure to follow the mixing instructions provided in the protocols.
- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of caps. Cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler and from other procedural steps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during thermal cycler incubation steps.
- In general, follow Biosafety Level 1 (BSL1) safety rules.
- Possible stopping points, where samples may be stored at 4°C or –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.

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

SureSelect XT HS2 DNA Kits and Supported Workflows

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

To determine the materials needed for your research design, first select the appropriate SureSelect XT HS2 DNA Kits for the Overnight-Hyb workflow from Table 2. Make sure that your selections include both Library Preparation and Target Enrichment reagents. Next, see Table 3 for a list of probes recommended for use in this workflow. If your research design includes target enrichment of SureSelect XT HS2 libraries with SureSelect XT HS Probes not listed in Table 3, see publication G9983-90000 for the recommended workflow.

Table 2 Ordering information for SureSelect XT HS2 DNA Kits for Overnight-Hyb, Post-capture pooling workflow

Agilent Part Number (Index Pairs Supplied)	Samples Processed	Library Prep Kit (Adaptor Type)	Target Enrichment Kit	SureSelect Streptavidin Beads
SureSelect XT HS2 DNA Library Preparation Kits for MBC-tagged Libraries (Target Enrichment Kit purchased separately)				
G9985A (Index 1–96) G9985B (Index 97–192) G9985C (Index 193–288) G9985D (Index 289–384)	96*	✓ (MBC-tagged)	✖ (order G9957B, below)	✖ Not Applicable
SureSelect XT HS2 DNA Library Preparation Kits for MBC-Free Libraries (Target Enrichment Kit purchased separately)				
G9956A (Index 1–96) G9956B (Index 97–192) G9956C (Index 193–288) G9956D (Index 289–384)	96*	✓ (MBC-Free)	✖ (order G9957B, below)	✖ Not Applicable
SureSelect XT HS2 Overnight-Hyb Target Enrichment Kit (Library Preparation Kit purchased separately)				
G9957B	96 Hybs*	✖ (order one of G9985A–D **OR** G9956A–D, above)	✓ (Overnight-Hyb, Post-Cap Pool)	✓

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

Table 3 Compatible Probes

Probe	Design ID	Part Number/Ordering Information	
Pre-designed Probes		**16 Reactions**	**96 Reactions**
SureSelect XT Human All Exon V8	S33266436	5191-6879	5191-6891
SureSelect XT Human All Exon V8+UTR	S33613367	Please contact Sales or your local representative for ordering information.	
SureSelect XT Human All Exon V8+NCV	S33700246		
SureSelect XT Human All Exon V7	S31285117	5191-4004	5191-4005
SureSelect XT Clinical Research Exome V4	S34226363	5280-0026	5280-0027
SureSelect XT Clinical Research Exome V2	S30409818	5190-9491	5190-9492
Custom Probes			
SureSelect Custom Tier1 1–499 kb	Please visit the SureDesign website to design Custom SureSelect XT probes and customized *Plus* content and to 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			
Agilent Community Designs: Please visit the Community Designs (NGS) webpage at agilent.com for information on custom panels developed in collaboration with experts in various fields.	Design details and ordering information are available at the SureDesign website on the *Published Designs* tab. Contact the SureSelect support team (see page 2) or your local representative if you need assistance.		

Additional Materials Required

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

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

Refer to Table 4 through Table 7 for additional materials needed to complete the protocols using the selected DNA sample type/fragmentation method.

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	Thermo Fisher Scientific p/n Q32850	—
Nuclease-free Water	Thermo Fisher Scientific p/n AM9930	Water should not be DEPC-treated
AMPure XP Kit 5 mL 60 mL	Beckman Coulter Genomics p/n A63880 p/n A63881	—

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 (see Table 5).

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 (flat or domed)*	Consult the thermal cycler manufacturer's recommendations
Nucleic acid analysis system (instrument and consumables)	Select one system from Table 7 on page 13
Qubit Fluorometer	Thermo Fisher Scientific p/n Q33238
Qubit Assay Tubes	Thermo Fisher Scientific p/n Q32856
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Microcentrifuge	Eppendorf microcentrifuge, model 5417C or equivalent
Plate or strip tube centrifuge	Labnet International MPS1000 Mini Plate Spinner, p/n C1000 (requires adapter, p/n C1000-ADAPT, for use with strip tubes) or equivalent
96-well plate mixer	Eppendorf ThermoMixer C, p/n 5382000023 and Eppendorf SmartBlock 96 PCR, p/n 5306000006, or equivalent
Magnetic separator	Thermo Fisher Scientific p/n 12331D or equivalent†
Vacuum concentrator‡	Savant SpeedVac, model DNA120, or equivalent
Multichannel and single channel pipettes	Rainin Pipet-Lite Multi Pipette or equivalent
Sterile, nuclease-free aerosol barrier pipette tips, vortex mixer, ice bucket, and powder-free gloves	General laboratory supplier

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

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

‡ Prepared libraries require dehydration prior hybridization, which requires 500–2000 ng library DNA in a 4 µL volume. If no vacuum concentrator is available in your laboratory, see *Troubleshooting* on page 76 for suggested protocol modifications.

Table 6 Additional Required Materials based on Sample Type/Fragmentation Method

Description	Vendor and Part Number	Usage 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	Recommended reagents for FFPE gDNA sample preparation prior to library preparation.
Deparaffinization Solution	Qiagen p/n 19093	
FFPE DNA integrity assessment system: Agilent NGS FFPE QC Kit 16 reactions 96 reactions OR TapeStation Genomic DNA Analysis Consumables: Genomic DNA ScreenTape Genomic DNA Reagents	 Agilent p/n G9700A p/n G9700B Agilent p/n 5067-5365 p/n 5067-5366	Recommended systems for FFPE gDNA qualification prior to library preparation.
DNA fragmentation options		
Mechanical DNA fragmentation system: Covaris Sample Preparation System Covaris microTUBE sample holders	 Covaris model E220 Covaris p/n 520045	Not required for workflows using enzymatic DNA fragmentation. Additional Covaris instrument models and sample holders may be used after optimization of shearing conditions.
SureSelect Enzymatic Fragmentation Kit	Agilent p/n 5191-4080 (96 reactions)	Not required for workflows using mechanical (Covaris-mediated) DNA shearing.

Table 7 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

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 46)
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

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Input DNA Preparation and Fragmentation

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

The library preparation protocol is compatible with both high-quality gDNA prepared from fresh or fresh-frozen samples and lower-quality DNA prepared from FFPE samples. Modifications required for FFPE samples are included throughout the protocol steps. For a summary of modifications for FFPE samples see Chapter 6, “Appendix 1: Using FFPE-derived DNA Samples” on page 53.

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

NOTE

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

Step 1. Prepare and qualify the genomic DNA samples

Preparation of high-quality gDNA from fresh biological samples

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

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

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

Additional qualification of DNA samples is not required for DNA derived from fresh biological samples. Proceed to "Step 2. Fragment the DNA" on page 17.

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

3 Assess the quality (DNA integrity) for each FFPE DNA sample using one of the methods below.

Option 1: Qualification using the Agilent NGS FFPE QC Kit

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

a Analyze a 1-µL aliquot of each FFPE gDNA sample using the Agilent NGS FFPE QC Kit. Follow the instructions provided in the kit user manual.

b Use the ΔΔCq score-based guidelines below (summarized in Table 9) to determine the appropriate input DNA quantification method for your sample:

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

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

Table 9 DNA input guidelines based on ΔΔCq DNA integrity score

ΔΔCq Score	DNA Input Guidelines
ΔΔCq≤1*	10 ng to 200 ng DNA, based on Qubit Assay quantification
ΔΔCq>1	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 the Agilent Genomic DNA ScreenTape assay DIN score

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

a Analyze a 1-µL aliquot of each FFPE gDNA sample using the Genomic DNA ScreenTape assay. Follow the instructions provided in the assay Quick Guide.

b Consult Table 10 for DIN score-based input DNA input guidelines.

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 with 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 a summary of shearing duration recommendations. Complete shearing instructions are also provided below.

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.

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

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

1 Prepare the DNA samples for the run by diluting 10–200 ng of each gDNA sample with 1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA) to a final volume of 50 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.

2 Complete the DNA shearing steps below for each gDNA sample.

- **a** Transfer the 50-µL DNA sample into a Covaris microTUBE.
- **b** Spin the microTUBE for 30 seconds to collect the liquid and to remove any bubbles from the bottom of the tube.

c Secure the microTUBE in the tube holder and shear the DNA with the settings in Table 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 (see two-round instructions below)	2 × 60 seconds (see two-round instructions below)	240 seconds
Bath Temperature	2° to 8° C	2° to 8° C	2° to 8° C

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

- Shear for 120 or 60 seconds (see Table 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

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

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

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

g After transferring the DNA sample, spin the microTUBE briefly to collect any residual sample volume. Transfer any additional collected liquid to the sample well used in step f.

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

The 50-µL sheared DNA samples are now ready for NGS sequencing library preparation, beginning with end repair/dA-tailing. Proceed to "Library Preparation using +/- MBC Adaptors" on page 21.

NOTE This is not a stopping point in the workflow, and analysis of the sheared samples is not required before they are used for library preparation. Proceed directly to page 21.

Method 2: Enzymatic DNA fragmentation

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

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

   If the DNA concentration is too low to supply 10–200 ng input DNA in 7 µL, sample volume may be reduced using a suitable concentration method. Alternatively, see *Troubleshooting* on page 75 for protocol modifications for dilute samples.

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

3 Preprogram a thermal cycler as shown in Table 13; pause until use in step 7. Optimal fragmentation conditions vary based on the NGS read length to be used in the workflow. Refer to Table 14 for the duration at 37°C appropriate for your sample type and required NGS read length.

Table 13 Thermal cycler program for enzymatic fragmentation (10 µL vol)

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

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

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

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

5 Add 3 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 samples in the thermal cycler and resume the enzymatic fragmentation 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 using +/- MBC Adaptors" on page 21.

NOTE This is not a stopping point in the workflow, and analysis of the enzymatically-fragmented samples is not required before they are used for library preparation. Proceed directly to page 21.

3
Library Preparation using +/- MBC Adaptors

Step 1. Prepare the ligation master mix 23
Step 2. Repair and dA-tail the DNA 3' ends 23
Step 3. Ligate the adaptor 24
Step 4. Purify libraries using AMPure XP Beads 25
Step 5. Amplify and index the pre-capture libraries 26
Step 6. Purify amplified libraries using AMPure XP Beads 28
Step 7. QC and quantify the pre-capture libraries 29

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

To process multiple samples, the protocol includes steps for preparation of reagent mixtures with overage, which are afterward distributed to the DNA library samples. Mixtures for preparation of 8 or 24 samples are shown in tables as examples.

The NGS library preparation protocol requires fragmented DNA samples produced by mechanical shearing (page 17 to page 18) or by enzymatic fragmentation (page 19 to page 20). Samples produced by either method should contain 10–200 ng of DNA fragments in a volume of 50 µL.

The library preparation protocols provided in this publication may also be used to prepare RNA sequencing libraries from the 50-µL samples containing cDNA fragments prepared according to the SureSelect XT HS2 RNA protocol. Protocol steps that require modification for RNA libraries are noted in the instructions on page 22 to page 27, with the modifications summarized in "Appendix 2: Protocol Modifications for RNA Libraries" on page 56.

This workflow segment uses the components listed in Table 16. Remove the listed reagents from cold storage, and prepare as directed before use (refer to the *Where Used* column).

Table 16 Reagents thawed before use in protocol

Storage Location	Kit Component	Preparative Steps	Where Used
For MBC-tagged libraries, get reagents from the *SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR)* box, stored at –20°C **For MBC-free libraries**, get reagents from the *SureSelect XT HS2 Library Preparation Kit for ILM, MBC-Free (Pre PCR)* box, stored at –20°C	Ligation Buffer (bottle)	Thaw on ice (may require >20 minutes) then keep on ice, vortex to mix	page 23
	T4 DNA Ligase (blue cap)	Place on ice just before use, invert to mix	page 23
	End Repair-A Tailing Buffer (bottle)	Thaw on ice (may require >20 minutes) then keep on ice, vortex to mix	page 24
	End Repair-A Tailing Enzyme Mix (orange cap)	Place on ice just before use, invert to mix	page 24
	For MBC-tagged libraries: XT HS2 Adaptor Oligo Mix (white cap) — **For MBC-free libraries**: SureSelect MBC-Free Adaptor Oligo Mix (black cap)	Thaw on ice then keep on ice, vortex to mix	page 25
	Herculase II Fusion DNA Polymerase (red cap)	Place on ice just before use, mix by pipetting	page 27
	5× Herculase II Buffer with dNTPs (clear cap)	Thaw on ice then keep on ice, vortex to mix	page 27
–20°C	SureSelect XT HS2 Index Primer Pairs for ILM (select the specific set of indexes to be used in the run): Index Pairs 1-96 (orange plate) Index Pairs 97-192 (blue plate) Index Pairs 193-288 (green plate) Index Pairs 289-384 (red plate)	Thaw on ice then keep on ice, vortex to mix	page 28
+4°C	AMPure XP Beads	Equilibrate at room temperature (RT) for at least 30 minutes before use, vortex to mix	page 25 and page 28

Additional preparative step for RNA library construction: To prepare RNA sequencing libraries from cDNA, dilute the provided XT HS2 Adaptor Oligo Mix or SureSelect MBC-Free Adaptor Oligo Mix 5-fold with 1X Low TE Buffer before use.

For a 96-reaction run, combine 100 µL of the appropriate Adaptor Oligo Mix and 400 µL of 1X Low TE Buffer. Vortex to mix and keep on ice until use in step 3 on page 25. Prepare a fresh dilution for each run.

Step 1. Prepare the ligation master mix

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

CAUTION The Ligation Buffer used in this step is viscous. Make sure to follow the mixing instructions in step 1 and step 2 below.

1 Vortex the thawed vial of Ligation Buffer for 15 seconds at high speed just before use.

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

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

Keep at room temperature for 30–45 minutes before use on page 24.

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 (bottle)	23 µL	207 µL	598 µL
T4 DNA Ligase (blue cap)	2 µL	18 µL	52 µL
Total	25 µL	225 µL	650 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 3' ends

CAUTION The End Repair-A Tailing Buffer used in this step is viscous. Make sure to follow the mixing instructions in step 2 and step 3 on page 24.

1 Preprogram a thermal cycler as shown in Table 18; pause until use in step 5.

Table 18 Thermal cycler program for end repair/dA-tailing (70 µl vol)

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

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.

3 Prepare the appropriate volume of dA-tailing master mix by combining the reagents in Table 19.

Slowly pipette the End Repair-A Tailing Buffer into a 1.5-mL tube, ensuring that the full volume is dispensed. Slowly add the End Repair-A Tailing Enzyme Mix, rinsing the enzyme tip with buffer solution after addition. Mix well by pipetting up and down 15–20 times with a pipette set to at least 80% of the mixture volume, or seal the tube and vortex at high speed for 5–10 seconds. Spin briefly and keep on ice.

Table 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 (bottle)	16 µL	144 µL	416 µL
End Repair-A Tailing Enzyme Mix (orange cap)	4 µL	36 µL	104 µL
Total	20 µL	180 µL	520 µL

4 Add 20 µL of the end repair/dA-tailing master mix to each sample well containing 50 µL of DNA fragments. 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 adaptor

1 Once the thermal cycling program in Table 18 reaches the 4°C Hold step, transfer the samples to ice. Preprogram the cycler as show in Table 20; pause until use in step 4.

Table 20 Thermal cycler program for ligation (100 µl vol)

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

2 To each end-repaired/dA-tailed DNA sample (approximately 70 µL), add 25 µL of the ligation master mix that was prepared on page 23 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.

3 Add 5 µL of the appropriate SureSelect Adaptor Oligo Mix to each sample:

- For **MBC-tagged** libraries–5 µL of XT HS2 Adaptor Oligo Mix (white-capped tube)
- For **MBC-free** libraries–5 µL of SureSelect MBC-Free Adaptor Oligo Mix (black-capped tube)

Mix by pipetting up and down 15–20 times using a pipette set to 70 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, mixing after each addition, as directed above.

For RNA library preparation from cDNA samples, make sure to dilute the appropriate Adaptor Oligo Mix 5-fold (see page 22) before use in this step.

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

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

Step 4. Purify libraries using AMPure XP Beads

Once the thermal cycler program in Table 20 reaches the 4°C hold step, purify the libraries using room-temperature AmpPure XP Beads.

Critical purification protocol parameters are summarized for experienced users in Table 21.

Table 21 AMPure XP bead cleanup parameters after adaptor ligation

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

1 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 all purification steps run on the same day.

2 Mix the room-temperature AMPure XP Beads well until homogeneous and consistent in color.

3 Transfer the DNA samples from the thermal cycler to room temperature, then add 80 µL of the bead suspension to each sample well.

4 Mix by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds then spin briefly to collect the samples, being careful not to pellet the beads.

5 Incubate the bead suspensions for 5 minutes at room temperature.

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

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

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

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

10 Repeat step 8 and step 9 once for a total of two washes.

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

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

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

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

13 Elute the library DNA by adding 35 µL of nuclease-free water to each sample well.

14 Mix by pipetting up and down 10–15 times or cap the wells and vortex at high speed for 5 seconds. Verify that all beads have been resuspended, with no visible clumps in the suspension or bead pellets retained on the sides of the wells. If samples were vortexed, spin briefly to collect the liquid, being careful not to pellet the beads.

15 Incubate for 2 minutes at room temperature.

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

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

Step 5. Amplify and index the pre-capture libraries

1 Determine the appropriate index pair assignment for each sample. See Table 61 on page 65 through Table 68 on page 72 for nucleotide sequences of the 8 bp index portion of the primers used to amplify the DNA libraries in this step.

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

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

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

2 Preprogram a thermal cycler as shown in Table 22; pause until use in step 6.

Table 22 Pre-capture PCR thermal cycler program (50 µl vol; heated lid ON)

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	See Table 23 for cycle number recommendations for DNA libraries. (For RNA library modifications, see page 57.)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

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	260 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	9 µL	26 µL
Total	11 µL	99 µL	286 µL

4 Add 11 µL of the PCR reaction mixture prepared in Table 24 to each sample well containing purified DNA library (34 µL).

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.

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

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

Step 6. Purify amplified libraries using AMPure XP Beads

Once the thermal cycler program in Table 22 reaches the 4°C hold step, purify the libraries using room-temperature AmpPure XP Beads.

Critical purification protocol parameters are summarized for experienced users in Table 25.

Table 25 AMPure XP bead cleanup parameters after pre-capture PCR

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

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

2 Mix the room-temperature AMPure XP Beads well until homogeneous and consistent in color.

3 Transfer the library DNA samples from the thermal cycler to room temperature, then add 50 µL of the bead suspension to each sample well.

4 Mix by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds then spin briefly to collect the samples, being careful not to pellet the beads.

5 Incubate the bead suspensions for 5 minutes at room temperature.

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

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

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

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

10 Repeat step 8 and step 9 once for a total of two washes.

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

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

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

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

13 Elute the library DNA by adding 15 µL of nuclease-free water to each sample well.

14 Mix by pipetting up and down 10–15 times or cap the wells and vortex at high speed for 5 seconds. Verify that all beads have been resuspended, with no visible clumps in the suspension or bead pellets retained on the sides of the wells. If samples were vortexed, spin briefly to collect the liquid, being careful not to pellet the beads.

15 Incubate for 2 minutes at room temperature.

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

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

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

Step 7. QC and quantify the pre-capture libraries

Analyze a sample of each library using one of the platforms listed in Table 26. Follow the instructions in the linked user guide provided for each assay.

Table 26 Pre-capture library analysis options

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

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 27 for fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided in Figure 2 through Figure 4 to illustrate typical results.

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

Table 27 Pre-capture library qualification guidelines

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

Observation of a low molecular weight peak, in addition to the expected library fragment peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to that seen in example electropherogram in Figure 4. See *Troubleshooting* on page 76 for additional considerations.

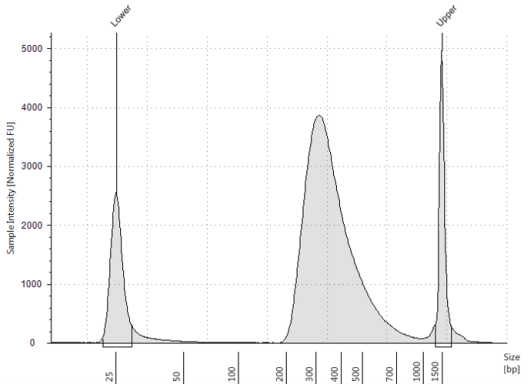

Figure 2 Pre-capture library prepared from a sheared high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

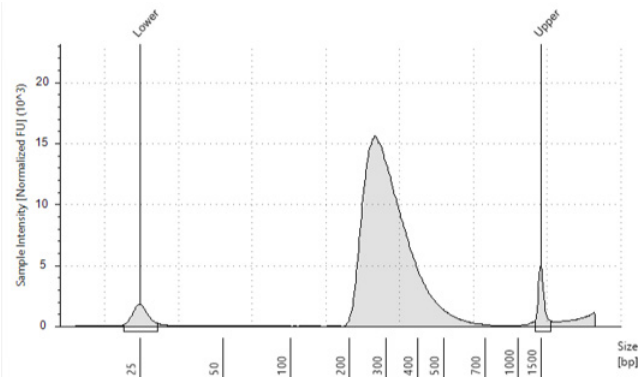

Figure 3 Pre-capture library prepared from a typical FFPE gDNA sample (fragmented by shearing) analyzed using a D1000 ScreenTape assay.

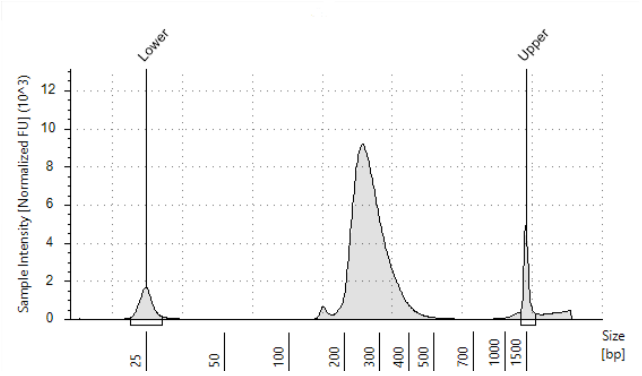

Figure 4 Pre-capture library prepared from a low-quality FFPE gDNA sample (fragmented by shearing) analyzed using a D1000 ScreenTape assay.

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

4
Hybridization, Capture, and Amplification

Step 1. Hybridize libraries to the SureSelect Probe 33
Step 2. Prepare streptavidin beads for capture 37
Step 3. Capture the hybridized libraries 37
Step 4. Amplify the captured libraries 39
Step 5. Purify the final libraries using AMPure XP Beads 41
Step 6. QC and quantify final libraries 42

In this workflow segment, the prepared gDNA libraries are hybridized with a target-specific probe using overnight hybridization conditions. After hybridization, the targeted molecules are captured on streptavidin beads and then PCR-amplified. Each DNA library sample is hybridized, captured, and amplified individually, prior to pooling for multiplex NGS.

The protocols provided in this section may also be used to prepare RNA sequencing libraries from prepared cDNA libraries. Protocol steps that require modification for RNA libraries are noted in the instructions on page 33 to page 38, with details provided in "Appendix 2: Protocol Modifications for RNA Libraries" on page 56.

The overnight hybridization workflow described in this section is optimal for SureSelect XT probe designs. SureSelect XT HS probe designs typically have improved performance using the Fast Hybridization, Post-capture Pooling workflow described in G9983-90000.

This workflow segment uses the components listed in Table 28. Remove the listed reagents from cold storage, where required, and prepare as directed before use (refer to the *Where Used* column).

Table 28 Reagents for Hybridization

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module Box 2 (Post PCR), stored at −20°C	SureSelect XT HS2 Blocker Mix (blue cap)	Thaw and keep on ice, vortex to mix	page 34
	SureSelect RNase Block (purple cap)	Thaw and keep on ice, vortex to mix	page 35
	SureSelect Hyb 3 (yellow cap)	Thaw and keep at RT, vortex to mix	page 33
SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module Box 1 (Post PCR), stored at RT	SureSelect Hyb 1 (bottle)	Keep at RT, vortex to mix	page 33
	SureSelect Hyb 2 (red cap)	Keep at RT, vortex to mix	page 33
	SureSelect Hyb 4 (black cap)	Keep at RT, vortex to mix	page 33
−80°C	SureSelect Probe	Thaw and keep on ice, vortex to mix	page 36

Step 1. Hybridize libraries to the SureSelect Probe

The hybridization reaction requires 500 to 2000 ng of prepared DNA library in a volume of 4 µL.

NOTE

Use the maximum amount of prepared DNA library available within the 500 to 2000 ng range to maximize complexity. This is especially important for libraries prepared from FFPE input DNA.

For RNA libraries, reduce the amount of prepared cDNA library used in step 1 below to 200 ng.

1 Place 500–2000 ng of each prepared library (maximum amount available within this range) into wells of a 96-well plate or strip tube. Keep on ice.

Using a vacuum concentrator at ≤ 45°C, dehydrate the prepped library samples to volume <4 µL without completely drying the samples. Measure the final volume in each well and then bring the volume of each sample to 4 µL with nuclease-free water.

NOTE

If a vacuum concentrator is not available in your laboratory, see *Troubleshooting* on page 76.

2 Prepare the Hybridization Buffer by mixing the components in Table 29 at room temperature.

If a precipitate forms, warm the Hybridization Buffer at 65°C for 5 minutes.

Keep the prepared Hybridization Buffer at room temperature until it is used in step 8.

Table 29 Preparation of Hybridization Buffer

Reagent	Volume for 1 reaction*	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
SureSelect Hyb 1 (bottle)	6.63 µL	66.3 µL	172.4 µL
SureSelect Hyb 2 (red cap)	0.27 µL	2.7 µL	7.0 µL
SureSelect Hyb 3 (yellow cap)	2.65 µL	26.5 µL	68.9 µL
SureSelect Hyb 4 (black cap)	3.45 µL	34.5 µL	89.7 µL
Total	13 µL	130 µL	338 µL

* Prepare Hybridization Buffer for at least 5 reaction equivalents per run to allow accurate pipetting volumes.

3 Refer to Table 30 to determine the optimal hybridization temperature for your probe. The pre-hybridization blocking step also uses the same temperature (see step 4).

Table 30 Hybridization temperature based on probe design

Probe Description	Optimal Blocking/Hybridization Temperature
SureSelect XT Human All Exon V8/V8+UTR/V8+NCV SureSelect XT Human All Exon V7 SureSelect XT Clinical Research Exome V4	67.5°C
All other XT probe designs	65°C

4 Preprogram a thermal cycler (heated lid ON) as shown in Table 31. Pause the program until samples are loaded in step 6.

Table 31 Pre-programmed thermal cycler program for DNA + Blocker incubation prior to hybridization

Segment Number	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	67.5°C or 65°C (see Table 30)	Hold (at least 5 minutes)

5 To each well containing 4 µL of prepared DNA library, 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.

6 Transfer the sealed sample plate or strip to the thermal cycler and resume the program in Table 31**.** Make sure that the DNA + Blocker Mix samples are held at 67.5°C or 65°C for at least 5 minutes before adding the remaining hybridization reaction components in step 9 below.

7 Prepare a 25% solution of SureSelect RNase Block (1 part RNase Block to 3 parts water) according to Table 32. Prepare the amount required for the number of hybridization reactions in the run, plus excess. Mix well and keep on ice.

Table 32 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 8, below, just before use. Keep the mixture at room temperature briefly until the mixture is added to the DNA samples in step 9. Do not keep solutions containing the probe at room temperature for extended periods.

8 Prepare the probe hybridization mix appropriate for your probe design size. Use Table 33 for probes ≥3 Mb or Table 34 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 9.

Table 33 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 7)	2 µL	18 µL	50 µL
SureSelect Probe (with design ≥ 3 Mb)	5 µL	45 µL	125 µL
SureSelect Hybridization Buffer (from step 2)	13 µL	117 µL	325 µL
Total	20 µL	180 µL	500 µL

Table 34 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 7)	2 µL	18 µL	50 µL
SureSelect Probe (with design < 3 Mb)	2 µL	18 µL	50 µL
SureSelect Hybridization Buffer (from step 2)	13 µL	117 µL	325 µL
Nuclease-free water	3 µL	27 µL	75 µL
Total	20 µL	180 µL	500 µL

9 Keeping the DNA + Blocker samples in the cycler held at 67.5°C or 65°C, transfer 20 µL of the room-temperature probe hybridization mix from step 8 to each sample well.

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

The hybridization reaction wells now contain approximately 29 µL.

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

11 Incubate the hybridization reactions in the thermal cycler (heated lid ON) at 67.5°C or 65°C (see Table 30) for 16 to 24 hours.

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 beads for capture

This workflow segment uses the components listed in Table 35. Remove the listed reagents from cold storage, where required, and prepare as directed before use (refer to the *Where Used* column).

Table 35 Reagents for Capture

Storage Location	Kit Component	Preparative Steps	Where Used
+4°C	SureSelect Streptavidin Beads (bottle)	Remove from 4°C just before use, vortex to mix	page 37
SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), stored at RT	SureSelect Binding Buffer (bottle)	Ready to use	page 37
	SureSelect Wash Buffer 1 (bottle)	Ready to use	page 38
	SureSelect Wash Buffer 2 (bottle)	Ready to use	page 38

1. Vigorously resuspend the vial of SureSelect 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 libraries

1. After the 16 to 24 hour hybridization incubation period is complete and after finishing all streptavidin bead preparation steps, transfer the samples to room temperature briefly.
2. Immediately transfer the entire volume (approximately 25–29 µL) of each hybridization mixture to wells containing 200 µL of washed streptavidin beads using a multichannel pipette.

   Pipette up and down 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 (approximately 1 to 2 minutes), 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 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 in the thermal cycler (heated lid ON) for 5 minutes at 70°C.

   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.

11 Keep the samples on ice until they are used in the PCR reactions below.

NOTE

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

Step 4. Amplify the captured libraries

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

Table 36 Reagents for post-capture amplification

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

1 Preprogram a thermal cycler as shown in Table 37; pause until use in step 5.

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

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	See Table 38 for cycle number recommendations for DNA libraries. (For RNA library modifications, see page 58.)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Table 38 Post-capture PCR cycle number recommendations

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

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

Table 39 Preparation of post-capture PCR reaction mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
Nuclease-free water	13 µL	117 µL	338 µL
5× Herculase II Buffer with dNTPs (clear cap)	10 µL	90 µL	260 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	9 µL	26 µL
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	9 µL	26 µL
Total	25 µL	225 µL	650 µL

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

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

5 Place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 37.

6 When the PCR amplification program is complete, spin the plate or strip tube briefly. Remove the streptavidin beads by placing the plate or strip tube on the magnetic stand at room temperature. Wait 2 minutes for the solution to clear.

7 **Transfer each supernatant (approximately 50 µL) to wells of a fresh plate or strip tube.** The streptavidin beads can be discarded at this time.

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

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

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

Step 5. Purify the final libraries using AMPure XP Beads

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

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

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

1. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 8.
2. Mix the room-temperature AMPure XP Beads well until homogeneous and consistent in color.
3. Add 50 µL of the bead suspension to each amplified DNA sample (approximately 50 µL) in the PCR plate or strip tube well.
4. Mix by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds then spin briefly to collect the samples, being careful not to pellet the beads.
5. Incubate the bead suspensions for 5 minutes at room temperature.
6. Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 2 to 5 minutes).
7. Keep the plate or strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 µL of fresh 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 and step 9 once for a total of two washes.
11. Cap the wells, then briefly spin the samples to collect the residual ethanol. Return the samples to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.

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

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

    Samples can instead be dried by keeping the unsealed plate or strip tube on the benchtop for approximately 5 minutes or until the residual ethanol has just evaporated.
13. Elute the library DNA by adding 25 µL of Low TE buffer to each sample well.
14. Mix by pipetting up and down 10–15 times or cap the wells and vortex at high speed for 5 seconds. Verify that all beads have been resuspended, with no visible clumps in the suspension or bead pellets retained on the sides of the wells. If samples were vortexed, spin briefly to collect the liquid, being careful not to pellet the beads.
15. Incubate for 2 minutes at room temperature.

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

17 Remove the cleared supernatant (approximately 25 µL) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

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

Step 6. QC and quantify final libraries

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

Table 41 Post-capture library analysis options

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

Each analysis method provides an electropherogram showing the size distribution of fragments in the sample and tools for determining the concentration of DNA in the sample. See Table 42 for fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided in Figure 5 through Figure 7 to illustrate typical results.

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

Table 42 Post-capture library qualification guidelines

NGS read length used for fragmentation protocol selection	Input DNA type	Expected DNA fragment size peak position
2 ×100 reads	Intact DNA	300 to 400 bp (see 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	330 to 450 bp
	FFPE DNA	200 to 450 bp

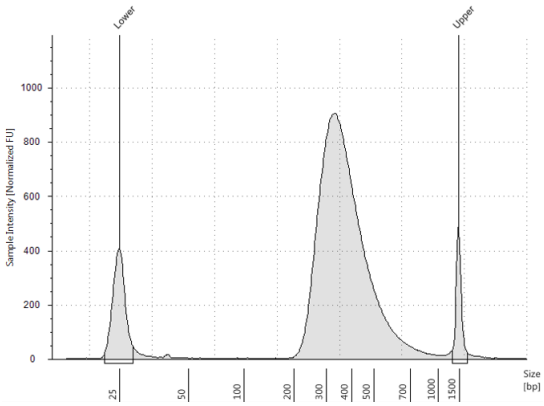

Figure 5 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

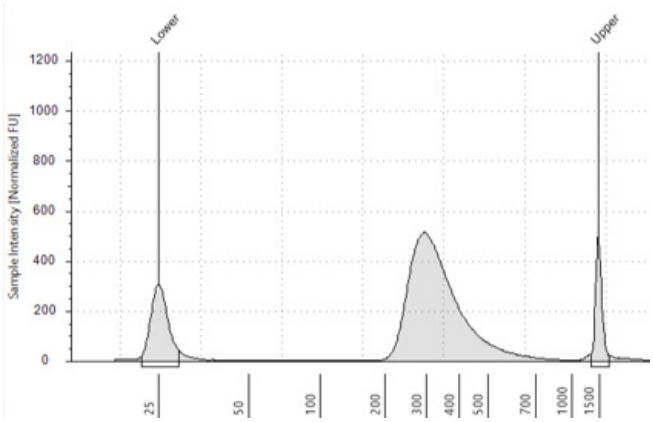

Figure 6 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

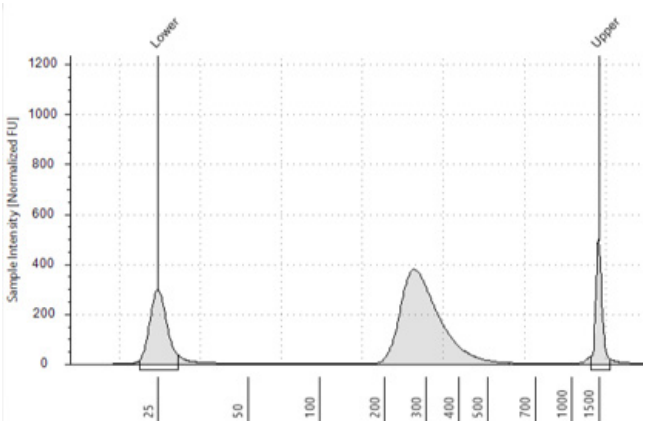

Figure 7 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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

5
NGS and Analysis Guidelines

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Step 4. Process and analyze the reads 49

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

Step 1. Pool samples for multiplexed sequencing

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the sequencer used, together with the amount of sequencing data required per sample 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 indexed library is present in equimolar amounts in the pool using one of the following methods. Use the diluent specified by your sequencing provider, such as Low TE, for the dilution steps.

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

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

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

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

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

is the number of indexes, and

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

Table 43 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM DNA.

Table 43 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 pool before sequencing, add Tween 20 to 0.1% v/v and store at –20°C short term, or store under the conditions specified by your sequencing provider.

Step 2. Prepare the sequencing samples

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

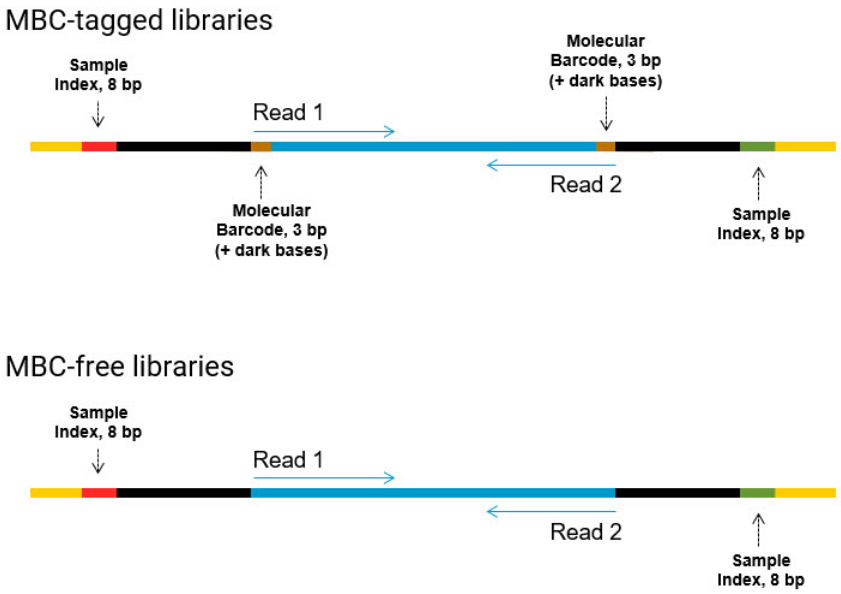

Figure 8 Content of SureSelect XT HS2 sequencing libraries. Each fragment contains one target insert (blue) surrounded by the following elements: Illumina paired-end sequencing elements (black), unique dual indexes (red and green), library PCR primers (yellow), and optional molecular barcodes (brown).

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

Table 44 Illumina Kit Configuration Selection Guidelines

Instrument	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
MiSeq	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	9–10 pM
MiSeq	All Runs	2 × 75 bp	150 Cycle Kit	v3	12–16 pM
iSeq 100	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	50–150 pM
NextSeq 500/550	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2.5	1.2–1.5 pM
NextSeq 1000/2000	All Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1, v2, or v3	650–1000 pM
HiSeq 4000	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1	300–400 pM
NovaSeq 6000	Standard Workflow Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1.0 or v1.5	300–600 pM
NovaSeq 6000	Xp Workflow Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1.0 or v1.5	200–400 pM
NovaSeq X	All runs	2 × 100 bp or 2 × 150 bp	100, 200 or 300 Cycle Kit	v1	90–180 pM

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

Step 3. Sequence the libraries

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

Table 45 Run settings for 2x150 bp sequencing

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

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

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

- Each of the sample-level indexes (i7 and i5) requires an 8-bp index read. For complete index sequence information, see Table 61 on page 65 through Table 68 on page 72.
- No custom primers are used for SureSelect XT HS2 library sequencing. Leave all *Custom Primers* options for *Read 1*, *Read 2*, *Index 1* and *Index 2* primers cleared/deselected during run setup.
- For MBC-tagged libraries, turn off any adaptor trimming tools included in Illumina's run setup and read processing software applications. Adaptors are trimmed in later processing steps using Agilent software tools to ensure proper processing of the adaptors, including the degenerate molecular barcodes (MBCs) in the adaptor sequences.
- For runs set up using Illumina's LRM, IEM, or BaseSpace applications, refer to Illumina's instructions and support resources for setting up runs with custom library prep kits and index kits in the selected software. For use in these applications, the SureSelect XT HS2 index sequences provided in Table 61 through Table 68 should be converted to .tsv/.csv file format or copied to a Sample Sheet according to Illumina's specifications for each application. If you need assistance with SureSelect XT HS2 run setup in your selected application (e.g., generating index files or Sample Sheet templates), contact the SureSelect support team (see page 2) or your local representative.

Step 4. Process and analyze the reads

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

- Demultiplex using Illumina's bcl2fastq, BCL Convert or DRAGEN software to generate paired end reads based on the dual indexes and remove sequences with incorrectly paired P5 and P7 indexes. For MBC-tagged libraries, turn off the MBC/UMI trimming options in Illumina's demultiplexing software to allow proper adaptor processing and use of the MBCs by Agilent's NGS software tools.
- The demultiplexed FASTQ data needs to be pre-processed to remove sequencing adaptors and extract the MBC sequences, if present. Both of the Agilent NGS software tools described below include the correct read pre-processing steps for SureSelect XT HS2 DNA libraries.
- Agilent's Alissa Reporter software provides a variety of applications for the complete FASTQ file to variant discovery process for SureSelect-enriched DNA libraries. See page 50 for more information.
- Agilent's Genomics NextGen Toolkit (AGeNT) software modules provide the tools to process the library read FASTQ files to analysis-ready BAM files for germline or somatic variant analysis workflows. See page 51 for more information.
- If you have prepared MBC-tagged libraries, but your sequence analysis pipeline excludes MBCs and is incompatible with Alissa Reporter and AGeNT software, you can trim or mask the first five bases from each read before alignment as described in the *Note* on page 52.

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

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

NOTE

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

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

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

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

Table 46 Alissa Reporter Application Chemistry selection based on Adaptor type

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

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

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

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

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

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

Agilent's AGeNT software is a Java-based toolkit used for SureSelect XT HS2 DNA library read processing steps. The AGeNT tools are designed to enable building, integrating, maintaining, and troubleshooting internal analysis pipelines for users with bioinformatics expertise. For additional information and to download this toolkit, visit the AGeNT page at www.agilent.com.

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

Table 48 Adaptor-based AGeNT workflow selection

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

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

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

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

NOTE

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

NOTE

If your libraries are MBC-tagged but your sequence analysis pipeline excludes MBCs, you can remove the first 5 bases from Read 1 and Read 2 by masking or trimming before proceeding to downstream analysis.

If demultiplexing using bcl2fastq, MBCs may be masked by including the base mask **N5Y*,I8,I8,N5Y*** (where * is replaced with the remaining read length after subtracting the 5 masked bases, e.g., use **N5Y146,I8,I8,N5Y146** for 2x150 NGS set up as shown in Table 45 on page 48). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

If demultiplexing using BCL Convert, MBCs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y*;I8;I8;N5Y*** (where * is replaced with read length after trimming, e.g., use **N5Y146;I8;I8;N5Y146** for 2x150 NGS set up as shown in Table 45 on page 48). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

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

6
Appendix 1: Using FFPE-derived DNA Samples

Protocol modifications for FFPE Samples 54
Methods for FFPE Sample Qualification 54
Sequencing Output Recommendations for FFPE Samples 55

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

Protocol modifications for FFPE Samples

Protocol modifications that should be applied to FFPE samples are summarized in Table 49.

Table 49 Summary of protocol modifications for FFPE samples

Workflow Step and page	Parameter	Condition for non-FFPE Samples	Condition for FFPE Samples
gDNA Sample Preparation page 15	Qualification of DNA Integrity	Not required	Required
DNA input for Library Preparation page 15	Input amount and means of quantification	10 ng to 200 ng, quantified by Qubit assay	Based on determined DNA integrity (see Table 9 and Table 10 on page 16)
DNA Shearing page 17	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 27	Cycle number	8–11	11–14
Sequencing page 55	Output augmentation	Per project requirements	1× to 10× based on determined DNA integrity (see Table 50 and Table 51 on page 55)

Methods for FFPE Sample Qualification

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

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include the precise quantity of amplifiable DNA in the sample to allow direct normalization of input DNA amount and a 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 50. 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 50 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 51. For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with DIN score of 4 requires approximately 200–400 Mb of sequencing output to achieve the same coverage.

Table 51 Recommended sequencing augmentation for FFPE-derived DNA samples

DIN value	Recommended fold increase for FFPE-derived sample
≥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

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Appendix 2: Protocol Modifications for RNA Libraries

Library preparation and pre-capture PCR protocol modifications 57
Hybridization and post-capture PCR protocol modifications 58
NGS resources for RNA libraries 59

This appendix provides a summary of the protocol modifications required to prepare target-enriched RNA sequencing libraries using the overnight hybridization, post-capture pooling workflow. Before you start, cDNA fragments should be prepared according to the SureSelect XT HS2 RNA protocol.

Most of the steps in the workflow are equivalent for DNA libraries prepared from gDNA fragments and RNA libraries prepared from cDNA fragments. To prepare RNA libraries using this workflow, follow the general protocol steps provided on page 22 to page 44, using the minor protocol modifications provided in this section on page 57 to page 59. This appendix also provides supplementary NGS resources for RNA libraries.

Library preparation and pre-capture PCR protocol modifications

- Dilute the XT HS2 Adaptor Oligo Mix or SureSelect MBC-Free Adaptor Oligo Mix 5-fold with 1X Low TE Buffer before use (see page 22).

  For a 96-reaction run, combine 100 µL of the appropriate Adaptor Oligo Mix and 400 µL of 1X Low TE Buffer. Vortex to mix and keep on ice until use. Prepare a fresh dilution for each run.
- When setting up the pre-capture PCR thermal cycler program on page 27, use the cycle number appropriate for your input RNA as detailed in Table 52 below.

Table 52 Pre-capture PCR cycle number recommendations

Quality of Input RNA	Quantity of Input RNA	Cycles
Intact RNA from fresh sample	100 to 200 ng	10 cycles
	50 ng	11 cycles
	10 ng	12 cycles
Good quality FFPE RNA (DV200 >50%)*	100 to 200 ng	12 cycles
	50 ng	13 cycles
	10 ng*	14 cycles
Poor quality FFPE RNA (DV200 20% to 50%)*	100 to 200 ng	13 cycles
	50 ng	14 cycles

* Qualify the FFPE-derived RNA as described in the SureSelect XT HS2 RNA protocol.

Hybridization and post-capture PCR protocol modifications

- The hybridization reaction requires 200 ng of prepared cDNA library in a volume of 4 µL.

  When setting up the hybridization reaction on page 33, add 200 ng of cDNA libraries to the hybridization wells in step 1. When required, use a vacuum concentrator to dehydrate the library samples to <4 µL as described on page 33. Adjust the volume of each sample to 4 µL with nuclease-free water.
- When setting up the post-capture PCR thermal cycler program on page 39, use the cycle number appropriate for your probe design as detailed in Table 53 below.

Table 53 Post-capture PCR cycle number recommendations

Probe Design Size	Cycles
Probes <0.2 Mb	16 cycles
Probes 0.2–3 Mb	14 cycles
Probes 3–5 Mb	13 cycles
Probes >5 Mb (including Human All Exon and Exome probes)	12 cycles

NGS resources for RNA libraries

Refer to "NGS and Analysis Guidelines" on page 45 for guidelines on sequencing the target-enriched libraries from library pooling through read pre-processing using the AGeNT Trimmer module.

The trimmed reads should be aligned using a suitable RNA data alignment tool. Once alignment is complete, the AGeNT CReaK (Consensus Read Kit) tool can be used in the single-strand consensus mode to generate consensus reads and mark or remove duplicates. The resulting BAM files are ready for downstream analysis including gene expression and variant discovery.

NOTE

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

Strandedness guidelines

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

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Reference

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

Reagent Kit Contents

Kits for the Library Prep (+/-MBCs), Overnight Hyb, Post-capture Pooling Workflow

The SureSelect XT HS2 DNA workflow supported in this publication uses the kits listed in Table 54. Detailed contents of each of the multi-part component kits listed in Table 54 are shown in Table 55 through Table 59 on the following pages.

Table 54 Kits Required for SureSelect XT HS2 Library Preparation with Overnight Hyb/Post-capture Pooling Workflow

SureSelect Library Type	Purchased Kits	Included Component Kits	Component Kit Part Numbers (specific index pairs included)	Storage Condition
MBC-tagged HS2 DNA libraries (96 Samples)	G9985A (Indexes 1-96), G9985B (Indexes 97-192), G9985C (Indexes 193-288), **OR** G9985D (Indexes 289-384)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR)	5500-0147	–20°C
		SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	5191-5688 (1-96), 5191-5689 (97-192), 5191-5690 (193-288), **OR** 5191-5691 (289-384)	–20°C
	G9957B (96 Hyb)	SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 1 (Post PCR)	5282-0051	RT
		SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 2 (Post PCR)	5282-0050	–20°C
		SureSelect Streptavidin Beads	5191-5742	+4°C
MBC-free HS2 DNA libraries (96 Samples)	G9956A (Indexes 1-96), G9956B (Indexes 97-192), G9956C (Indexes 193-288), **OR** G9956D (Indexes 289-384)	SureSelect XT HS2 Library Preparation Kit for ILM, MBC-Free (Pre PCR)	5282-0052	–20°C
		SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	5191-5688 (1-96), 5191-5689 (97-192), 5191-5690 (193-288), **OR** 5191-5691 (289-384)	–20°C
	G9957B (96 Hyb)	SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 1 (Post PCR)	5282-0051	RT
		SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 2 (Post PCR)	5282-0050	–20°C
		SureSelect Streptavidin Beads	5191-5742	+4°C

Component Kit Details

Table 55 SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR) content

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

Table 56 SureSelect XT HS2 Library Preparation Kit for ILM, MBC-Free (Pre PCR) content

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

Table 57 SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) content

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

* See page 64 through page 72 for index pair sequence information; see page 73 for index plate position maps.

Table 58 SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 1 (Post PCR) content

Kit Component	96 Hyb Kit (p/n 5282-0051)
SureSelect Hyb 1	bottle
SureSelect Hyb 2	tube with red cap
SureSelect Hyb 4	tube with black cap
SureSelect Binding Buffer	bottle
SureSelect Wash Buffer 1	bottle
SureSelect Wash Buffer 2	bottle

Table 59 SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 2 (Post PCR) content

Kit Component	96 Hyb Kit (p/n 5282-0050)
SureSelect Hyb 3	tube with yellow cap
SureSelect XT HS2 Blocker Mix	tube with blue cap
SureSelect RNase Block	tube with purple cap
SureSelect Post-Capture Primer Mix	tube with clear cap
Herculase II Fusion DNA Polymerase	tube with red cap
5× Herculase II Reaction Buffer with dNTPs	tube with clear cap

SureSelect XT HS2 Index Primer Pair Information

The SureSelect XT HS2 Index Primer Pairs are provided pre-combined. Each member of the primer pair contains a unique 8-bp P5 or P7 index, resulting in dual-indexed NGS libraries. Each well contains a single-use aliquot of a specific pair of forward plus reverse primers. One primer pair is provided in each well of a 96-well plate (see page 73 for plate maps).

The nucleotide sequence of the index portion of each primer is provided in Table 61 on page 65 through Table 68 on page 72. P7 indexes are shown in forward orientation, applicable to any of the supported Illumina platforms. P5 indexes are shown in two orientations (forward and reverse complement) for use with different platforms and sequencing run setup and management tools, e.g., Local Run Manager and Instrument Run Setup. Illumina sequencing platforms and their P5 sequencing orientation are shown in Table 60. Correct representation of the P5 index orientation in sample sheets or during sequencing run setup is crucial to successful demultiplexing. Refer to Illumina support documentation and resources to determine the correct P5 index orientation for your application.

Table 60 P5 index sequencing orientation by Illumina platform

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

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

SureSelect XT HS2 Index Primer Pair Sequences

Table 61 SureSelect XT HS2 Index Primer Pairs 1–48, provided in orange 96-well plate

Primer Pair #	Index Strip	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Index Strip	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
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 62 SureSelect XT HS2 Index Primer Pairs 49–96, provided in orange 96-well plate

Primer Pair #	Index Strip	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Index Strip	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
49	A07	TGAGGAGT	CGCGCAAT	ATTGCGCG	73	A10	AACGCATT	ATAGTGAC	GTCACTAT
50	B07	ATGACGAA	TGAGGAGT	ACTCCTCA	74	B10	CAGTTGCG	AACGCATT	AATGCGTT
51	C07	TACGGCGA	ATGACGAA	TTCGTCAT	75	C10	TGCCTCGA	CAGTTGCG	CGCAACTG
52	D07	AGCGAGTT	TACGGCGA	TCGCCGTA	76	D10	AAGGCTTA	TGCCTCGA	TCGAGGCA
53	E07	TGTATCAC	AGCGAGTT	AACTCGCT	77	E10	GCAATGAA	AAGGCTTA	TAAGCCTT
54	F07	GATCGCCT	TGTATCAC	GTGATACA	78	F10	AAGAACCT	GCAATGAA	TTCATTGC
55	G07	GACTCAAT	GATCGCCT	AGGCGATC	79	G10	CTGTGCCT	AAGAACCT	AGGTTCTT
56	H07	CAGCTTGC	GACTCAAT	ATTGAGTC	80	H10	TACGTAGC	CTGTGCCT	AGGCACAG
57	A08	AGCTGAAG	CAGCTTGC	GCAAGCTG	81	A11	AAGTGGAC	TACGTAGC	GCTACGTA
58	B08	ATTCCGTG	AGCTGAAG	CTTCAGCT	82	B11	CAACCGTG	AAGTGGAC	GTCCACTT
59	C08	TATGCCGC	ATTCCGTG	CACGGAAT	83	C11	CTGTTGTT	CAACCGTG	CACGGTTG
60	D08	TCAGCTCA	TATGCCGC	GCGGCATA	84	D11	GCACGATG	CTGTTGTT	AACAACAG
61	E08	AACTGCAA	TCAGCTCA	TGAGCTGA	85	E11	GTACGGAC	GCACGATG	CATCGTGC
62	F08	ATTAGGAG	AACTGCAA	TTGCAGTT	86	F11	CTCCAAGC	GTACGGAC	GTCCGTAC
63	G08	CAGCAATA	ATTAGGAG	CTCCTAAT	87	G11	TAGTCTGA	CTCCAAGC	GCTTGGAG
64	H08	GCCAAGCT	CAGCAATA	TATTGCTG	88	H11	TTCGCCGT	TAGTCTGA	TCAGACTA
65	A09	TCCGTTAA	GCCAAGCT	AGCTTGGC	89	A12	GAACTAAG	ATACGAAG	CTTCGTAT
66	B09	GTGCAACG	TCCGTTAA	TTAACGGA	90	B12	AAGCCATC	GAGATTCA	TGAATCTC
67	C09	AGTAACGC	GTGCAACG	CGTTGCAC	91	C12	AACTCTTG	AAGCCATC	GATGGCTT
68	D09	CATAGCCA	AGTAACGC	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 63 SureSelect XT HS2 Index Primer Pairs 97–144, provided in blue 96-well plate

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

Table 64 SureSelect XT HS2 Index Primer Pairs 145–192, provided in blue 96-well plate

Primer Pair #	Index Strip	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Index Strip	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
145	A07	TGTGACTA	CTCCGGTT	AACCGGAG	169	A10	CGCTCAGA	CTAACAAG	CTTGTTAG
146	B07	GCTTCCAG	TGTGACTA	TAGTCACA	170	B10	TAACGACA	CGCTCAGA	TCTGAGCG
147	C07	CATCCTGT	GCTTCCAG	CTGGAAGC	171	C10	CATACTTG	TAACGACA	TGTCGTTA
148	D07	GTAATACG	CATCCTGT	ACAGGATG	172	D10	AGATACGA	CATACTTG	CAAGTATG
149	E07	GCCAACAA	GTAATACG	CGTATTAC	173	E10	AATCCGAC	AGATACGA	TCGTATCT
150	F07	CATGACAC	GCCAACAA	TTGTTGGC	174	F10	TGAAGTAC	AATCCGAC	GTCGGATT
151	G07	TGCAATGC	CATGACAC	GTGTCATG	175	G10	CGAATCAT	TGAAGTAC	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 65 SureSelect XT HS2 Index Primer Pairs 193–240, provided in green 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
193	A01	GTCTCTTC	GAAGCCTC	GAGGCTTC	217	A04	GCGGTATG	CACGAGCT	AGCTCGTG
194	B01	AGTCACTT	GTCTCTTC	GAAGAGAC	218	B04	TCTATGCG	GCGGTATG	CATACCGC
195	C01	AGCATACA	AGTCACTT	AAGTGACT	219	C04	AGGTGAGA	TCTATGCG	CGCATAGA
196	D01	TCAGACAA	AGCATACA	TGTATGCT	220	D04	CACAACTT	AGGTGAGA	TCTCACCT
197	E01	TTGGAGAA	TCAGACAA	TTGTCTGA	221	E04	TTGTGTAC	CACAACTT	AAGTTGTG
198	F01	TTAACGTG	TTGGAGAA	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 66 SureSelect XT HS2 Index Primer Pairs 241–288, provided in green 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
241	A07	AAGAGACA	TGCTTACG	CGTAAGCA	265	A10	CAATGCTG	CATGAATG	CATTCATG
242	B07	TAGCTATG	AAGAGACA	TGTCTCTT	266	B10	CTTGATCA	CAATGCTG	CAGCATTG
243	C07	TCTGCTAC	TAGCTATG	CATAGCTA	267	C10	GCGAATTA	CTTGATCA	TGATCAAG
244	D07	GTCACAGA	TCTGCTAC	GTAGCAGA	268	D10	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 67 SureSelect XT HS2 Index Primer Pairs 289–336, provided in red 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
289	A01	AGATAGTG	GATGAGAT	ATCTCATC	313	A04	AGCTACAT	GATCCATG	CATGGATC
290	B01	AGAGGTTA	AGATAGTG	CACTATCT	314	B04	CGCTGTAA	AGCTACAT	ATGTAGCT
291	C01	CTGACCGT	AGAGGTTA	TAACCTCT	315	C04	CACTACCG	CGCTGTAA	TTACAGCG
292	D01	GCATGGAG	CTGACCGT	ACGGTCAG	316	D04	GCTCACGA	CACTACCG	CGGTAGTG
293	E01	CTGCCTTA	GCATGGAG	CTCCATGC	317	E04	TGGCTTAG	GCTCACGA	TCGTGAGC
294	F01	GCGTCACT	CTGCCTTA	TAAGGCAG	318	F04	TCCAGACG	TGGCTTAG	CTAAGCCA
295	G01	GCGATTAC	GCGTCACT	AGTGACGC	319	G04	AGTGGCAT	TCCAGACG	CGTCTGGA
296	H01	TCACCACG	GCGATTAC	GTAATCGC	320	H04	TGTACCGA	AGTGGCAT	ATGCCACT
297	A02	AGACCTGA	TCACCACG	CGTGGTGA	321	A05	AAGACTAC	TGTACCGA	TCGGTACA
298	B02	GCCGATAT	AGACCTGA	TCAGGTCT	322	B05	TGCCGTTA	AAGACTAC	GTAGTCTT
299	C02	CTTATTGC	GCCGATAT	ATATCGGC	323	C05	TTGGATCT	TGCCGTTA	TAACGGCA
300	D02	CGATACCT	CTTATTGC	GCAATAAG	324	D05	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 68 SureSelect XT HS2 Index Primer Pairs 337–384, provided in red 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
337	A07	TGAGACGC	GCCTTCAT	ATGAAGGC	361	A10	CTGAGCTA	GCACAGTA	TACTGTGC
338	B07	CATCGGAA	TGAGACGC	GCGTCTCA	362	B10	CTTGCGAT	CTGAGCTA	TAGCTCAG
339	C07	TAGGACAT	CATCGGAA	TTCCGATG	363	C10	GAAGTAGT	CTTGCGAT	ATCGCAAG
340	D07	AACACAAG	TAGGACAT	ATGTCCTA	364	D10	GTTATCGA	GAAGTAGT	ACTACTTC
341	E07	TTCGACTC	AACACAAG	CTTGTGTT	365	E10	TGTCGTCG	GTTATCGA	TCGATAAC
342	F07	GTCGGTAA	TTCGACTC	GAGTCGAA	366	F10	CGTAACTG	TGTCGTCG	CGACGACA
343	G07	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 Plate Maps

Table 69 through Table 72 show the plate 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 69 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 70 Plate map for SureSelect XT HS2 Index Primer Pairs 97-192, provided in blue plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Table 71 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 72 Plate map for SureSelect XT HS2 Index Primer Pairs 289-384, provided in red plate

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

Troubleshooting Guide

If recovery of gDNA from samples is low

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

If concentration of DNA samples is too low for enzymatic fragmentation

- ✔ The standard enzymatic fragmentation protocol requires 10–200 ng DNA in a volume of 7 µL, and uses a final fragmentation reaction volume of 10 µL. For dilute samples, enzymatic fragmentation may be performed in a reaction volume of 20 µL using the modified protocol below:
  - Bring samples containing 10–200 ng DNA to 17 µL final volume with 1X Low TE Buffer in plate or strip sample wells.
  - Prepare the Fragmentation master mix as directed in Table 15 on page 19.
  - Add 3 µL of the master mix to each DNA sample well. Mix and spin as directed on page 20.
  - Run the thermal cycling program in Table 13 on page 19 using the 37°C fragmentation duration shown in the table below.

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

If yield of pre-capture libraries is low

- ✔ The library preparation protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance of the highly viscous buffer and enzyme solutions used in the protocol. Be sure to adhere to all instructions when setting up the reactions.
- ✔ Ensure that the ligation master mix (see page 23) is kept at room temperature for 30–45 minutes before use.
- ✔ PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the pre-capture PCR cycle number by 1 to 2 cycles. If a high molecular weight peak (>500 bp) is observed in the electropherogram for a sample with low yield, the DNA may be overamplified. Repeat library preparation for the sample, decreasing the pre-capture PCR cycle number by 1 to 3 cycles.
- ✔ DNA isolated from FFPE tissue samples may be over-fragmented or have modifications that adversely affect library preparation processes. Use the Agilent NGS FFPE QC Kit to determine the precise quantity of amplifiable DNA in the sample and allow direct normalization of input DNA amount.
- ✔ Yield from the AMPure XP Bead purification steps may be suboptimal. Consider the factors below:
  - Adhere to all of the bead and reagent handling steps in the protocol. In particular, make sure to equilibrate beads at room temperature for at least 30 minutes before use, and use freshly-prepared 70% ethanol (prepared on day of use) for each purification procedure.

- Ensure that the AMPure XP Beads are not over-dried just prior to sample elution (see step 12 on page 26 and page 29). If you are drying the beads at 37°C for 2 minutes, consider using drying conditions of RT for 5 minutes.
- Increasing the elution incubation time (up to 10 minutes) may improve recovery, especially for longer DNA fragments.

If solids observed in the End Repair-A Tailing Buffer

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

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

✔ Shearing may not be optimal. For intact, high-quality DNA samples, ensure that shearing is completed using the two-round shearing protocol provided, including all spinning and vortexing steps.

✔ Any bubbles present on the microTUBE filament may disrupt complete shearing. Spin the microTUBE for 30 seconds before the first round of shearing to ensure that any bubbles are released.

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

✔ FFPE DNA pre-capture libraries may have a smaller fragment size distribution due to the presence of DNA fragments in the sample input that are smaller than the targeted post-fragmentation size. Adhere to the DNA quality guidelines provided on page 15.

✔ 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 28.

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

✔ The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to the samples analyzed on page 31. 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 24. 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.

✔ When preparing MBC-free RNA sequencing libraries, the SureSelect MBC-Free Adaptor Oligo Mix provided in this kit should be diluted 5-fold before use (see page 22). Use of the undiluted adaptor solution may increase the quantity of adaptor-dimers in the cDNA library.

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

✔ The overnight hybridization protocol requires 4 µL samples containing the maximum amount of library DNA available in the 500–2000 ng range, using a vacuum concentrator to reduce the volumes of the prepared library samples. If a vacuum concentrator is not available in your laboratory, consider using one of the protocol modifications below to generate more concentrated library samples for hybridization. Both modifications may result in some loss of library complexity; method 1 is the recommended solution with lower risk of complexity losses.

- Method 1 (recommended): In step 13 on page 29, reduce the amount of nuclease-free water used for elution from 15 µL to 6 µL for routine overnight-hybridization workflow runs in your laboratory.

- Method 2: If prepared libraries were already eluted in 15 µL using the instructions on page 28 to page 29, libraries can be concentrated using an additional round of AMPure XP Bead purification. Use a bead volume of 1.8X the available library sample volume in step 3, then elute using 6 µL of nuclease-free water in step 13.

If yield of post-capture libraries is low

- ✔ PCR cycle number may require optimization. Repeat library preparation and target enrichment for the sample, increasing the post-capture PCR cycle number by 1 to 2 cycles.
- ✔ The probe used for hybridization may have been compromised. Verify the expiration date on the probe vial or Certificate of Analysis. Adhere to the recommended storage and handling conditions. Ensure that the probe hybridization mix is prepared immediately before use, as directed on page 36, and that solutions containing the probe are not held at room temperature for extended periods.
- ✔ Yield from the AMPure XP Bead purification step may be suboptimal. Consider the factors below:
  - Adhere to all of the bead and reagent handling steps in the protocol. In particular, make sure to equilibrate beads at room temperature for at least 30 minutes before use, and use freshly-prepared 70% ethanol (prepared on day of use) for each purification procedure.
  - Ensure that the AMPure XP Beads are not over-dried just prior to sample elution (see step 12 on page 41). If you are drying the beads at 37°C for 2 minutes, consider using drying conditions of RT for 5 minutes.
  - Increasing the elution incubation time (up to 10 minutes) may improve recovery, especially for longer DNA fragments.

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

- ✔ DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color and verify that you are using the bead volume recommended for post-capture purification on page 41.

If low percent on target is observed in library sequencing results

- ✔ Stringency of post-hybridization washes may have been lower than required. Complete the wash steps as directed, paying special attention to the details of SureSelect Wash Buffer 2 washes listed below:
  - Ensure that SureSelect Wash Buffer 2 is pre-warmed to 70°C before use (see page 38). Select a thermal cycler with a block configured for efficient heating of 0.2 mL liquid volumes; ensure that the plasticware containing the wash buffer is fully seated in the thermal cycler block wells, with minimal liquid volume visible above the block during the pre-warming step.
  - Samples are maintained at 70°C during washes (see page 38)
  - Bead suspensions are mixed thoroughly during washes by pipetting up and down **and** vortexing (see page 38)
- ✔ 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 67.5°C or 65°C sample temperature during mixing and transfer steps (step 10 to step 11 on page 36).

Quick Reference Protocol

An abbreviated summary of the protocol steps is provided below for experienced users. Use the complete protocol on page 15 to page 44 until you are familiar with all of the protocol details such as reagent mixing instructions and instrument settings. When preparing RNA sequencing libraries, refer to page 57 and page 58 for a brief list of protocol modifications.

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 15 and page 16. Mechanically shear DNA using Covaris with shearing conditions on page 18 OR enzymatically fragment DNA using SureSelect Enzymatic Fragmentation Kit with protocol on page 19-page 20. Either method yields fragmented DNA in final sample volume of 50 µL
Prepare Ligation master mix	Per 24 reactions: 598 µL Ligation Buffer + 52 µL T4 DNA Ligase Keep at room temperature 30–45 min before use
Prepare End-Repair/dA-Tailing master mix	Per 24 reactions: 416 µL End Repair-A Tailing Buffer + 104 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 **OR** 5 µL SureSelect XT HS2 MBC-Free Adaptor Oligo Mix Incubate in thermal cycler: 30 min @ 20°C, Hold @ 4°C
Purify DNA	100 µL DNA sample + 80 µL AMPure XP bead suspension Elute DNA in 35 µL nuclease-free H_2O, removing 34 µL to fresh well
Prepare PCR master mix	Per 24 reactions: 260 µL 5× Herculase II Reaction Buffer with dNTPs + 26 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 27
Purify amplified DNA	50 µL amplified DNA + 50 µL AMPure XP bead suspension Elute DNA in 15 µL nuclease-free H_2O
Quantify and qualify DNA	Analyze quantity and quality using TapeStation, Bioanalyzer, or Fragment Analyzer System
	Hybridization/Capture
Prep DNA in hyb plate	Dry 500–2000 ng of each purified prepared library in well using vacuum concentrator at <45°C Resuspend each library in 4 µL nuclease-free H_2O
Prep Overnight Hyb Buffer	Per 24 reactions: 172.4 µL SureSelect Hyb 1 + 7.0 µL SureSelect Hyb 2 + 68.9 µL SureSelect Hyb 3 + 89.7 µL SureSelect Hyb 4 Keep at RT
Program thermal cycler	Input the thermal cycler program appropriate for your probe design using 65°C or 67.5°C incubation temperature, as shown in Table 31 on page 34 Pause program

Step	Summary of Conditions
Run pre-hybridization blocking protocol	4 µL library DNA + 5 µL SureSelect XT HS2 Blocker Mix Run paused thermal cycler program Keep at 65°C/67.5°C hold at least 5 min (see Table 31) before Hyb Mix addition below
Prepare Hyb Mix	Prepare 25% RNAse Block dilution, then prepare appropriate Probe Hyb Mix below (amounts shown are for 24 reactions): Probes ≥3 Mb: 50 µL 25% RNase Block + 125 µL Probe + 325 µL Overnight Hyb Buffer Probes <3 Mb: 50 µL 25% RNase Block + 50 µL Probe + 75 µL nuclease-free H_2O + 325 µL Overnight Hyb Buffer
Run the hybridization	With cycler held at 65°C/67.5°C and samples retained in cycler, add 20 µL Probe Hyb Mix to wells Incubate in the thermal cycler at 65°C/67.5°C for 16 to 24 hours
Prepare streptavidin beads	Wash 50 µL Streptavidin T1 beads 3 × in 200 µL SureSelect Binding Buffer Resuspend washed beads in 200 µL SureSelect Binding Buffer
Capture hybridized libraries	Add hybridized samples (~29 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 24 reactions: 338 µL nuclease-free H_2O+ 260 µL 5× Herculase II Reaction Buffer with dNTPs + 26 µL SureSelect Post-Capture Primer Mix + 26 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 39
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 provides instructions for using SureSelect HS2 DNA Reagent Kits with an Overnight-Hyb, Post-capture pooling workflow.

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