SureSelect$^{XT\ HS}$ / SureSelect^{XT} Low Input Target Enrichment with Pre-Capture Pooling

Preparation of Multiplexed NGS Libraries for the Illumina Sequencing Platform

Protocol

Version E0, September 2022

SureSelect platform manufactured with Agilent SurePrint Technology

For Research Use Only. Not for use in diagnostic procedures.

Agilent Technologies

Notices

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In this Guide...

This guide provides an optimized protocol for preparation of pre-capture pooled NGS sequencing libraries, prepared using the SureSelect$^{XT\ HS}$/SureSelect^{XT} Low Input System with a Pre-Capture Probe Library.

1 Before You Begin

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

2 Preparation and Fragmentation of Input DNA

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

3 Library Preparation

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

4 Hybridization and Capture

This chapter describes the steps to pool, then hybridize and capture the prepared DNA libraries using a SureSelect or ClearSeq Probe.

5 Post-Capture Sample Processing for Multiplexed Sequencing

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

6 Appendix: Using FFPE-derived DNA Samples

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

7 Reference

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

What's New in Version E0

- Support for SureSelect XT HS Human All Exon V8+UTR and SureSelect XT HS Human All Exon V8+NCV Probes. See Table 2 on page 13 for ordering information.
- Design ID information added to Table 2 on page 13 for pre-designed SureSelect probes.
- Updates to tube cap strip recommendations from domed caps to flat/domed caps based on specific cycler recommendations (see Table 5 on page 16 and related updates on page 11, Table 7 on page 19, and page 55).
- Update to enzymatic fragmentation instructions in step 1 on page 28 for optional use of 1X Low TE Buffer as solvent for DNA samples.
- Updates to recommended reagent volumes for 24 reaction runs in Table 15 on page 33 and Table 17 on page 35.
- New AmpPure XP bead purification protocol parameter summary tables for experienced users (see Table 19 on page 37, Table 24 on page 42, and Table 36 on page 63).
- New *Note* on cross-platform index equivalence on page 39.
- Updates to downstream sequencing support information (see page 72 to page 84). Key updates include guidelines for demultiplexing using Illumina's BCL Convert software (see page 74) and support for Agilent's new CReaK tool, replacing the LocatIt tool in AGeNT v3.0 (see page 84).
- Support for use of Agilent's Alissa Reporter software for SureSelect XT HS DNA library sequence pre-processing and human germline DNA variant analysis (see page 75 and page 83).
- Update to *Troubleshooting* on page 98 on thermal cycler block configuration requirements for efficient heating of SureSelect Wash Buffer 2.
- Update to "Notice to Purchaser" on page 2.

What's New in Version D0

- Support for SureSelect XT HS PreCap Human All Exon V8 Probe. See Table 2 on page 13 for ordering information and see page 50 for pre-capture pooling recommendations. See related updates to the *Quick Reference Protocol* on page 101.
- Updates to the "Hybridization and Capture" chapter on page 49 through page 56, including updates to Table 27 on page 52 and additional minor updates throughout the chapter.
- Update to *Note* on page 25 and new footnote to Table 12 on page 29 on impacts of initial FFPE DNA sample fragment size on final library fragment size distribution.

Content

1 Before You Begin

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

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

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

Overview of the Workflow

The workflow for SureSelect$^{XT\ HS}$/SureSelect^{XT} Low Input NGS library preparation using pre-capture pooling is summarized in Figure 1.

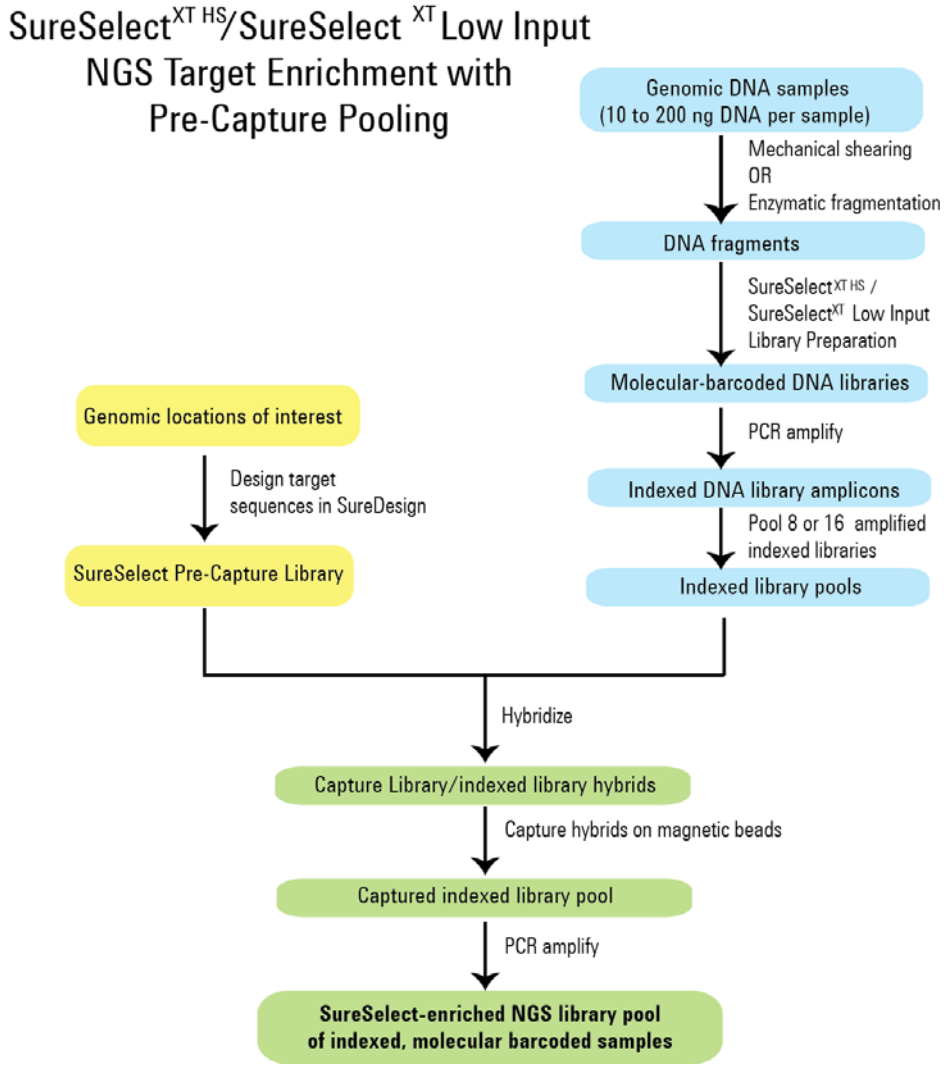


Figure 1 Overall target-enriched sequencing sample preparation workflow.

Procedural Notes

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

Safety Notes

CAUTION

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

Materials Required

Materials required to complete the SureSelect^{XT} Low Input Pre-capture Pooling 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

All SureSelect^{XT} Low Input Pre-capture Pooling workflows require the reagents listed in Table 1 and a compatible target enrichment Probe selected from Table 2 or Table 3. Refer to Table 4 through Table 6 for additional materials needed to complete the protocols according to your DNA sample type/fragmentation method.

Table 1 SureSelect Reagents for Pre-capture Pooling Workflows

Description	96 Reaction Kit Part Number*
SureSelect XT Low Input Library Preparation Kit for ILM (Pre PCR) with Index 1-96, 96 Reactions	Agilent p/n G9916A†
SureSelect XT HS Target Enrichment Kit ILM Hyb Module (Post PCR), 16 Reactions (sufficient for 96 pre-capture pooled samples)	Agilent p/n G9916B

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

† Dual-indexed libraries, prepared using Agilent p/n G9916A and SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM (p/n 5191-4056) and prepared according to the *Sample Preparation* instructions in Agilent publication G9703-90050, are also compatible with the pre-capture pooling and hybridization protocols in this publication.

Table 2 Compatible Pre-designed Probes for Pre-capture Pooling

Probe Description		Part Number
SureSelect XT HS PreCap Human All Exon V8 (12 Hybs)*	S33266340	5191-6877
SureSelect XT HS PreCap Human All Exon V8+UTR (12 Hybs)*	S33613271	5191-7405
SureSelect XT HS PreCap Human All Exon V8+NCV (12 Hybs)*	S33699751	5191-7411
SureSelect XT HS PreCap Human All Exon V7 (12 Hybs)*	S31285117	5191-5735
SureSelect XT2 Human All Exon V6 (12 Hybs)*	S07604514	5190-8873
SureSelect XT2 Human All Exon V6 + UTRs (12 Hybs)*	S07604624	5190-8885
SureSelect XT2 Human All Exon V6 + COSMIC (12 Hybs)*	S07604715	5190-9311
SureSelect XT2 Mouse All Exon (12 Hybs)*	S0276129	5190-4682
SureSelect XT2 Clinical Research Exome V2 (12 Hybs)*	S30409818	5190-9501
ClearSeq Inherited Disease XT2 (12 Hybs)*	S0684402	5190-7525
ClearSeq Comprehensive Cancer XT2 (6 Hybs)†	0425761	5190-8018

* The 12-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 8 samples/pool (for 12 hybs × 8 samples/hyb). Contains enough reagent for 2 runs with 6 Hybridization reactions per run using the run setup on page 54.

† The 6-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 16 samples/pool (for 6 hybs × 16 samples/hyb). Contains enough reagent for 1 run with 6 Hybridization reactions per run using the run setup on page 54.

Table 3 Compatible Custom and Plus Probes for Pre-capture Pooling

Probe Description	Ordering Information
Custom Probes*	
SSEL PreCap Custom Tier1 1–499 kb (6 Hybs or 30 Hybs)†	Please visit the SureDesign website to design Custom SureSelect probes and obtain ordering information. Contact the SureSelect support team (see page 2) or your local representative if you need assistance.
SSEL PreCap Custom Tier2 0.5 –2.9 Mb (6 Hybs or 30 Hybs)†	
SSEL PreCap Custom Tier3 3 –5.9 Mb (6 Hybs or 30 Hybs)†	
SSEL PreCap Custom Tier4 6 –11.9 Mb (6 Hybs or 30 Hybs)†	
SSEL PreCap Custom Tier5 12–24 Mb (6 Hybs or 30 Hybs)†	
Pre-designed Probes customized with additional *Plus* custom content	
SureSelect XT2 Human All Exon V6 Plus 1 (12 Hybs)‡	Please visit the SureDesign website to design the customized *Plus* content and obtain ordering information. Contact the SureSelect support team (see page 2) or your local representative if you need assistance.
SureSelect XT2 Human All Exon V6 Plus 2 (12 Hybs)‡	
SureSelect XT2 Clinical Research Exome V2 Plus 1 (12 Hybs)‡	
SureSelect XT2 Clinical Research Exome V2 Plus 2 (12 Hybs)‡	
ClearSeq Comprehensive Cancer Plus XT2 (6 Hybs)†	
ClearSeq Inherited Disease Plus XT2 (12 Hybs)‡	

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

† The 6-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 16 samples/pool (for 6 hybs × 16 samples/hyb). Contains enough reagent for 1 run with 6 Hybridization reactions using the run setup on page 54. The 30-Hyb Probes support target enrichment for 480 samples pooled using the configuration recommended for the included designs of 16 samples/pool (for 30 hybs × 16 samples/hyb). Contains enough reagent for 5 runs with 6 Hybridization reactions per run using the run setup on page 54.

‡ The 12-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 8 samples/pool (for 12 hybs × 8 samples/hyb). Contains enough reagent for 2 runs with 6 Hybridization reactions per run using the run setup on page 54.

Table 4 Required Reagents--All Sample Types/Fragmentation Methods

Description	Vendor and part number
AMPure XP Kit 5 ml 60 ml 450 ml	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
Dynabeads MyOne Streptavidin T1 2 ml 10 ml 50 ml	Thermo Fisher Scientific p/n 65601 p/n 65602 p/n 65604D
1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276
Qubit BR dsDNA Assay Kit 100 assays 500 assays	Thermo Fisher Scientific p/n Q32850 p/n Q32853
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

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

Table 5 Required Equipment--All Sample Types/Fragmentation Methods

Description	Vendor and part number
Thermal Cycler with 96-well, 0.2 ml block	Various suppliers
Plasticware compatible with the selected thermal cycler: 96-well plates or 8-well strip tubes Tube cap strips (flat or domed, based on cycler/lid requirements)*	Consult the thermal cycler manufacturer's recommendations
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
Small-volume spectrophotometer	NanoDrop 2000, Thermo Fisher Scientific p/n ND-2000 or equivalent
Vacuum concentrator	Savant SpeedVac, model DNA120, or equivalent
Multichannel pipette	Rainin Pipet-Lite Multi Pipette or equivalent
Single channel pipettes (10-, 20-, 200-, and 1000-µl capacity)	Rainin Pipet-Lite Pipettes or equivalent
Sterile, nuclease-free aerosol barrier pipette tips	General laboratory supplier
Vortex mixer	General laboratory supplier

Table 5 Required Equipment--All Sample Types/Fragmentation Methods

Description	Vendor and part number
DNA Analysis Platform and Consumables†	
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
OR	
Agilent 4200/4150 TapeStation	Agilent p/n G2991AA/G2992AA
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585
Magnetic separator	Thermo Fisher Scientific p/n 12331D or equivalent‡
Ice bucket	general laboratory supplier
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.

† DNA samples may also be analyzed using the Agilent 5200 Fragment Analyzer, p/n M5310AA, and associated NGS Fragment Kits (DNF-473-0500 and DNF-474-0500). Implement any sample dilution instructions provided in protocols in this document, and then follow the assay instructions provided for each NGS Fragment Kit.

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

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

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

Optional Materials

Table 7 Supplier Information for Optional Materials

Description	Vendor and Part Number	Purpose
Tween 20	Sigma-Aldrich p/n P9416-50ML	Sequencing library storage (see page 73)
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

2 Preparation and Fragmentation of Input DNA

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

The library preparation protocol is compatible with both high-quality gDNA prepared from fresh or fresh-frozen samples and lower-quality DNA prepared from FFPE samples. Modifications required for FFPE samples are included throughout the protocol steps. For a summary of modifications for FFPE samples see Chapter 6, “Appendix: Using FFPE-derived DNA Samples” on page 85.

The 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. Analysis using the molecular barcodes is recommended when sample is available in low amounts (10–50 ng) or when detecting very low allele frequency variants using small probe designs.

Step 1. Prepare and analyze quality of genomic DNA samples

Preparation of high-quality gDNA from fresh biological samples

1 Prepare high-quality gDNA using a suitable purification system, such as Qiagen's QIAamp DNA Mini Kit, following the manufacturer's protocol. The protocol requires 10 ng to 200 ng DNA input.

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

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

Additional qualification of DNA samples is not required for DNA derived from fresh biological samples. Proceed to "Step 2. Fragment the DNA" on page 25.

Preparation and qualification of gDNA from FFPE samples

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

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

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

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

Option 1: Qualification using the Agilent NGS FFPE QC Kit (Recommended Method)

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include a ΔΔCq DNA integrity score and the precise quantity of amplifiable DNA in the sample, allowing direct normalization of DNA input for each sample. DNA input recommendations based on ΔΔCq scores for individual samples are summarized in Table 8.

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

b Remove a 1 µl aliquot of the FFPE gDNA sample for analysis using the Agilent NGS FFPE QC Kit to determine the ΔΔCq DNA integrity score. See the kit user manual (G9700-90000) at www.agilent.com for more information.

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

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

Table 8 SureSelect XT Low Input DNA input modifications based on ΔΔCq DNA integrity score

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

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

Option 2: Qualification using Agilent's Genomic DNA ScreenTape assay DIN score

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

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

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

c Using the DIN score reported for each sample in the Genomic DNA ScreenTape assay, consult Table 9 to determine the recommended amount of input DNA for the sample.

Table 9 SureSelect XT Low Input DNA input modifications based on DNA Integrity Number (DIN) score

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

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

Step 2. Fragment the DNA

Method 1: Mechanical DNA Shearing using Covaris

In this step, 50-µl gDNA samples are sheared using conditions optimized for either high-quality or FFPE DNA. The target DNA fragment size is 150 to 200 bp.

NOTE

This protocol has been optimized using a Covaris model E220 instrument and the 130-µl Covaris microTUBE for a target DNA fragment size of 150 to 200 bp. If you wish to use a different Covaris instrument model/sample holder or if your NGS workflow requires a different DNA fragment size (e.g., for translocation detection with the SureSelect Cancer All-In-One assay), consult the manufacturer's recommendations for shearing conditions for the recommended DNA fragment size.

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. All FFPE samples should be sheared for 240 seconds (see Table 10 on page 26) 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.

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

   a Check that the water in the Covaris tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate in use.

   b Check that the water covers the visible glass part of the tube.

   c On the instrument control panel, push the Degas button. Degas the instrument according to the manufacturer's recommendations, typically 30–60 minutes.

   d Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C. Consult the manufacturer's recommendations for addition of coolant fluids to prevent freezing.

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

NOTE

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

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

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

b Spin the microTUBE for 30 seconds to collect the liquid and to remove any bubbles from the bottom of the tube.

c Secure the microTUBE in the tube holder and shear the DNA with the settings in Table 10.

Table 10 Shear settings for Covaris E-series instrument (SonoLab software v7 or later)

Setting	High-quality DNA	FFPE DNA
Duty Factor	10%	10%
Peak Incident Power (PIP)	175	175
Cycles per Burst	200	200
Treatment Time	2 × 120 seconds	240 seconds
Bath Temperature	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 seconds
- 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 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" on page 31.

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

Method 2: Enzymatic DNA Fragmentation

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

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

   If the DNA concentration is too low to supply the 10–200 ng input amount required for your workflow in 7 µl, sample volume may be reduced using a suitable concentration method. Alternatively, see *Troubleshooting* on page 95 for protocol modifications for dilute samples.

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

3 Preprogram a thermal cycler (with the heated lid ON) with the program in Table 11. Immediately pause the program, and keep paused until samples are loaded in step 7.

Table 11 Thermal cycler program for enzymatic fragmentation*

Step	Temperature	Time
Step 1	37°C	Varies–see Table 12
Step 2	65°C	5 minutes
Step 3	4°C	Hold

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

Optimal fragmentation conditions may vary based on the NGS read length to be used in the workflow. Refer to Table 12 on page 29 for the duration at 37°C appropriate for your sample type and required NGS read length.

Table 12 Fragmentation duration based on sample type and NGS read length

NGS read length requirement	Target fragment size	Duration of 37°C incubation step (Table 11)	
		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 13.

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 13 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 (blue cap)	2 µl	18 µl	50 µl
SureSelect Fragmentation Enzyme (green cap)	1 µl	9 µl	25 µl
Total	3 µl	27 µl	75 µl

5 Add 3 µl of the Fragmentation master mix to each sample well containing 7 µl of input DNA.

6 Mix well by pipetting up and down 20 times or cap the wells and vortex at high speed for 5–10 seconds. Spin the samples briefly.

7 Immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 11.

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

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

NOTE

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

3 Library Preparation

The library preparation protocol is used to prepare DNA libraries for sequencing using the Illumina paired-read platform. For each sample to be sequenced, an individual indexed and molecular-barcoded library is prepared. For an overview of the workflow, see Figure 1 on page 10.

NOTE

Dual-indexed libraries, prepared using the *Library Preparation* instructions in Agilent publication G9703-90050, are also compatible with the pre-capture pooling and hybridization protocols in this publication.

The NGS library preparation protocol that begins here is used for fragmented DNA samples produced by mechanical shearing (as detailed on page 25 to page 27) or produced by enzymatic fragmentation (as detailed on page 28 to page 30). Samples produced by either method should contain 10–200 ng of DNA fragments in a volume of 50 µl.

Step 1. Repair and dA-Tail the DNA ends

Protocol steps in this section use the components listed inTable 14. Thaw and mix each component as directed in Table 14 before use.

Remove the AMPure XP beads from cold storage and equilibrate to room temperature in preparation for use on page 37. *Do not freeze the beads at any time.*

Table 14 Reagents thawed before use in protocol

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

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

1 Before starting the end-repair protocol, prepare the Ligation master mix to allow equilibration to room temperature before use.

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

CAUTION

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

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

   b Prepare the appropriate volume of Ligation master mix by combining the reagents in Table 15.

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

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

Table 15 Preparation of Ligation master mix

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

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

2 Preprogram a thermal cycler (with the heated lid ON) with the program in Table 16 for the End Repair and dA-Tailing steps. Immediately pause the program, and keep paused until samples are loaded in step 6.

Table 16 Thermal cycler program for End Repair/dA-Tailing*

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

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

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

CAUTION

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

4 Prepare the appropriate volume of End Repair/dA-Tailing master mix, by combining the reagents in Table 17.

Slowly pipette the End Repair-A Tailing Buffer into a 1.5-ml Eppendorf tube, ensuring that the full volume is dispensed. Slowly add the End Repair-A Tailing Enzyme Mix, rinsing the enzyme tip with buffer

solution after addition. Mix well by pipetting up and down 15–20 times or seal the tube and vortex at high speed for 5–10 seconds. Spin briefly to collect the liquid and keep on ice.

Table 17 Preparation of End Repair/dA-Tailing master mix

Reagent	Volume for 1 reaction	Volume for 24 reactions (includes excess)
End Repair-A Tailing Buffer (bottle)	16 µl	416 µl
End Repair-A Tailing Enzyme Mix (orange cap)	4 µl	104 µl
Total	**20 µl**	**520 µl**

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

6 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 16.

Step 2. Ligate the molecular-barcoded adaptor

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

2 Preprogram a thermal cycler (with the heated lid ON) for the Ligation step with the program in Table 18. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 18 Thermal cycler program for Ligation*

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

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

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

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

NOTE Make sure to add the Ligation master mix and the Adaptor Oligo Mix to the samples in separate addition steps as directed in step 3 and step 4 above, mixing after each addition.

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

NOTE A unique molecular barcode sequence is incorporated into each library DNA fragment at this step.

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

Step 3. Purify the sample using AMPure XP beads

In this step, the DNA libraries are purified using AmpPure XP Beads. Critical purification protocol parameters are summarized for experienced users in Table 19.

Table 19 AMPure XP bead cleanup parameters after adaptor ligation

Parameter	Value
Volume of RT AMPure XP bead suspension added to each sample well	80 µl
Final elution solvent and volume	35 µl nuclease-free water
Amount of eluted sample transferred to fresh well	Approximately 34.5 µl

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

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

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

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

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

5 Incubate samples for 5 minutes at room temperature.

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

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

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

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

10 Repeat step 8 to step 9 once.

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

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

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

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

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

15 Incubate for 2 minutes at room temperature.

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

17 Remove the cleared supernatant (approximately 34.5 µl) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

NOTE It may not be possible to recover the entire 34.5-µl supernatant volume at this step; transfer the maximum possible amount of supernatant for further processing. To maximize recovery, transfer the cleared supernatant to a fresh well in two rounds of pipetting, using a P20 pipette set at 17.25 µl.

Step 4. Amplify the adaptor-ligated library

This step uses the components listed in Table 20. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 20 Reagents for pre-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Pipette up and down 15–20 times	page 41
5× Herculase II Reaction Buffer (clear cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	page 41
100 mM dNTP Mix (green cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	page 41
Forward Primer (brown cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	page 41
SureSelect XT Low Input Index Primers	SureSelect XT Low Input Index Primers for ILM (Pre PCR),* –20°C	Vortexing	page 41

* Indexing primers are provided in a yellow 96-well plate labeled *Index Plate 1* containing indexes 1–96.

1 Determine the appropriate index assignments for each sample. See page 94 for more information on the index primers used to amplify the DNA libraries in this step.

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

NOTE Take care to avoid combining libraries with the same index sequence when multiplexing libraries prepared using different SureSelect kit formats. For example, indexes 1-32 in SureSelect XT Low Input Reagent Kits (provided in yellow plate) are equivalent to indexes A01-H04 in SureSelect XT HS Reagent Kits (provided in black capped-tubes) and to indexes A01-H04 in Magnis SureSelect XT HS automation kits (provided in black index strips).

CAUTION The SureSelect XT Low Input Index Primers are provided in single-use aliquots. To avoid cross-contamination of libraries, use each well for a single library preparation reaction. Do not re-use any residual volume for subsequent experiments.

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

Table 21 Pre-Capture PCR Thermal Cycler Program*

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8 to 14, based on input DNA quality and quantity (see Table 22)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

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

Table 22 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 23, on ice. Mix well on a vortex mixer.

Table 23 Preparation of Pre-Capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 24 reactions (includes excess)
5× Herculase II Reaction Buffer (clear cap)	10 µl	250 µl
100 mM dNTP Mix (green cap)	0.5 µl	12.5 µl
Forward Primer (brown cap)	2 µl	50 µl
Herculase II Fusion DNA Polymerase (red cap)	1 µl	25 µl
Total	**13.5 µl**	**337.5 µl**

4 Add 13.5 µl of the PCR reaction mixture prepared in Table 23 to each purified DNA library sample (34.5 µl) in the PCR plate wells.

5 Add 2 µl of the appropriate SureSelect XT Low Input Index Primer 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 release any bubbles.

6 Before adding the samples to the thermal cycler, resume the program in Table 21 to bring the temperature of the thermal block to 98°C. Once the cycler has reached 98°C, immediately place the sample plate or strip tube in the thermal block and close the lid.

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

Step 5. Purify the amplified library with AMPure XP beads

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

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

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

1. Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use.
2. Prepare 400 µl of 70% ethanol per sample, plus excess, for use in step 8.
3. Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.
4. Add 50 µl of homogeneous AMPure XP beads to each 50-µl amplification reaction in the PCR plate or strip tube. Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 minutes).
7. Keep the plate or strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 µl of freshly-prepared 70% ethanol into each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 and step 9 step once.

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

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

13 Add 15 µl nuclease-free water to each sample well.

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

15 Incubate for 2 minutes at room temperature.

16 Put the plate or strip tube in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.

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

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

Step 6. Assess quality and quantity

Sample analysis can be done with either the 2100 Bioanalyzer instrument or an Agilent TapeStation instrument.

NOTE

Using either analysis method, observation of a low molecular weight peak, in addition to the expected library fragment peak, indicates the presence of adaptor-dimers in the library. Adaptor-dimer removal is not required for libraries that will be target-enriched in later steps of the workflow. However, for libraries being prepared for whole-genome sequencing (not specifically supported by this user guide), samples with an adaptor-dimer peak must be subjected to an additional round of SPRI-purification. To complete, first dilute the sample to 50 µl with nuclease free water, then follow the SPRI purification procedure on page 42.

Option 1: Analysis using the 2100 Bioanalyzer instrument and DNA 1000 Assay

Use a Bioanalyzer DNA 1000 chip and reagent kit. Perform the assay according to the Agilent DNA 1000 Kit Guide.

1. Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µl of each sample for the analysis. Load the prepared chip into the instrument and start the run within five minutes after preparation.
3. Verify that the electropherogram shows the peak of DNA fragment size positioned between 300 to 400 bp for high-quality DNA and approximately 200 to 400 bp for FFPE DNA. Sample electropherograms are shown in Figure 2 (library prepared from high-quality DNA), Figure 3 (library prepared from medium-quality FFPE DNA), and Figure 4 (library prepared from low-quality FFPE DNA).

   The appearance of an additional low molecular weight 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 sample electropherograms on page 45. See Troubleshooting information on page 97 for additional considerations.
4. Determine the concentration of each library by integrating under the entire peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

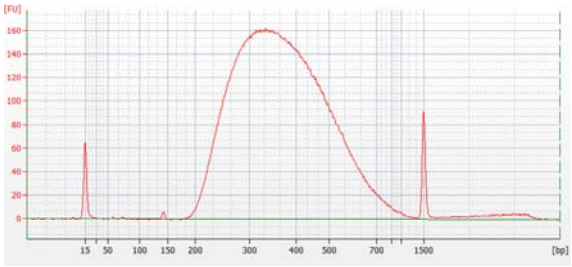


Figure 2 Pre-capture library prepared from a high-quality gDNA sample analyzed using a DNA 1000 Bioanalyzer assay.

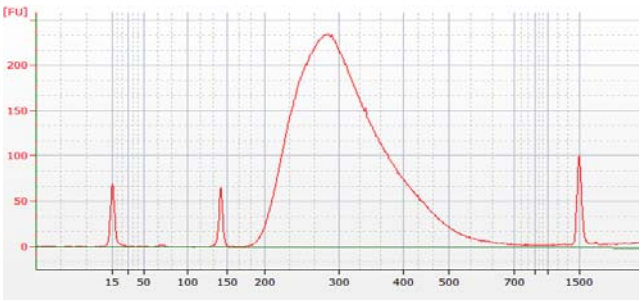


Figure 3 Pre-capture library prepared from a typical FFPE gDNA sample analyzed using a DNA 1000 Bioanalyzer assay.

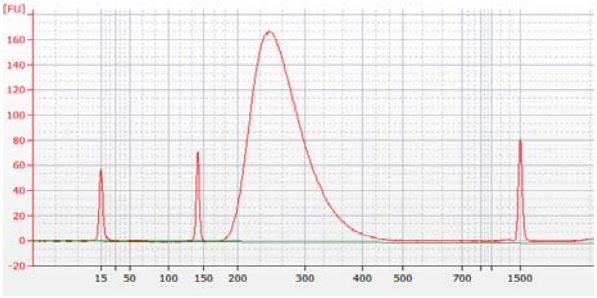


Figure 4 Pre-capture library prepared from a low-quality FFPE gDNA sample analyzed using a DNA 1000 Bioanalyzer assay.

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.

Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and associated reagent kit. For more information to do this step, see the Agilent D1000 Assay Quick Guide.

1 Prepare the TapeStation samples as instructed in the reagent kit guide. Use 1 µl of each DNA sample diluted with 3 µl of D1000 sample buffer for the analysis.

CAUTION For accurate quantitation, make sure to thoroughly mix the combined DNA and sample buffer by vortexing the assay plate or tube strip for 1 minute on the IKA MS3 vortex mixer provided with the 4200/4150 TapeStation system before loading the samples.

2 Load the sample plate or tube strips from step 1, the D1000 ScreenTape, and loading tips into the TapeStation as instructed in the reagent kit guide. Start the run.

3 Verify that the electropherogram shows the peak of DNA fragment size positioned between 300 to 400 bp for high-quality DNA and approximately 200 to 400 bp for FFPE DNA. Sample electropherograms are shown in Figure 5 (library prepared from high-quality DNA), Figure 6 (library prepared from medium-quality FFPE DNA), and Figure 7 (library prepared from low-quality FFPE DNA).

   The appearance of an additional low molecular weight 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 sample electropherograms on page 47 to page 48. See Troubleshooting information on page 97 for additional considerations.

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

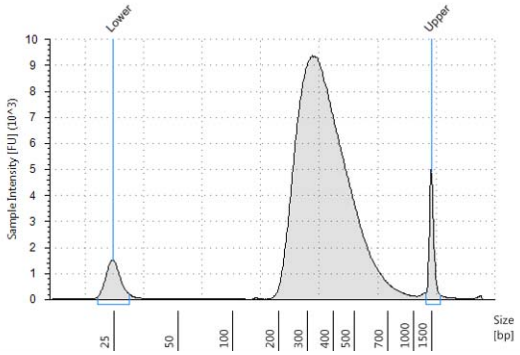


Figure 5 Pre-capture library prepared from a high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

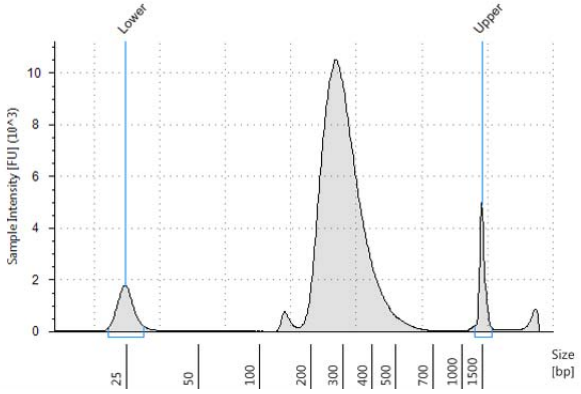


Figure 6 Pre-capture library prepared from a typical FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

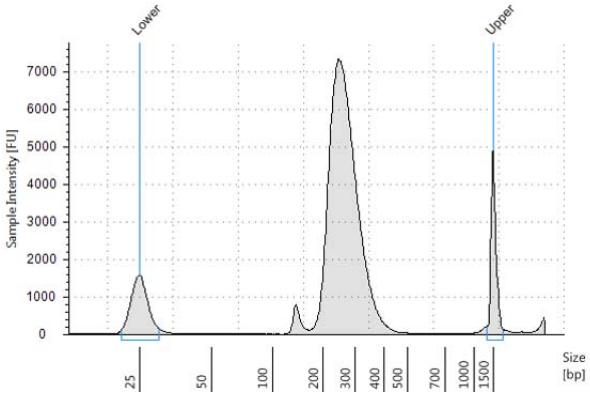


Figure 7 Pre-capture library prepared from a low-quality FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

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 and Capture

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

Recommendations for the total amount of library DNA pooled per hybridization reaction and the number of indexes combined per pool vary for different probes. See Table 25 for pooling recommendations.

The standard single-day protocol includes the hybridization step (approximately 90 minutes) immediately followed by capture and amplification steps. If required, the hybridized samples may be held overnight with capture and amplification steps completed the following day by using the simple protocol modifications noted on page 52.

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

Step 1. Pool indexed DNA samples for hybridization

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

Each hybridization reaction requires a total of either 3 µg or 1.5 µg indexed gDNA, made up of equal amounts of 8 or 16 individual libraries, based on the specific probe design used for hybridization. See Table 25 for probe-specific gDNA library pool composition recommendations.

Table 25 Pre-capture pooling recommendations

Probe description	Total amount of indexed gDNA pool used for hybridization	Number of indexed gDNA libraries per pool	Amount of each gDNA library in pool
SureSelect XT HS PreCap Human All Exon V8	3 µg	8	375 ng
SureSelect XT HS PreCap Human All Exon V7	1.5 µg	8	187.5 ng
SureSelect XT2 Mouse All-Exon	1.5 µg	8	187.5 ng
SureSelect Clinical Research Exome	1.5 µg	8	187.5 ng
SureSelect Focused Exome	1.5 µg	8	187.5 ng
ClearSeq Inherited Disease	1.5 µg	8	187.5 ng
ClearSeq Comprehensive Cancer	1.5 µg	16	93.75 ng
SureSelect Custom Probes	1.5 µg	16	93.75 ng

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

   Each final capture reaction pool should contain either 3 µg or 1.5 µg indexed gDNA (see Table 25).

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

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

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

4. Cap the wells, then vortex the strip tube or plate vigorously for 30 seconds. Spin in a centrifuge or mini-plate spinner to collect the liquid, then keep the samples on ice until use on page 52.

Step 2. Hybridize DNA samples to the probe

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

Table 26 Reagents for Hybridization

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect XT HS and XT Low Input Blocker Mix (blue cap)	SureSelect XT HS Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	page 52
SureSelect RNase Block (purple cap)	SureSelect XT HS Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	page 53
SureSelect Fast Hybridization Buffer (bottle)	SureSelect XT HS Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw and keep at Room Temperature	page 54
Probe	–80°C	Thaw on ice	page 54

1 Preprogram a thermal cycler (with the heated lid ON) with the program in Table 27. Immediately pause the program, and keep paused until samples are loaded in step 3.

Table 27 Pre-programmed thermal cycler program for Hybridization*

Segment #	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	65°C	10 minutes
3	1	65°C	1 minute (Pause cycler here for reagent addition; see step 6 on page 55)
4	60	65°C†	1 minute
		37°C	3 seconds
5	1	65°C†	Hold briefly until ready to begin capture steps on page 57

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

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

NOTE

The Hybridization thermal cycling program in Table 27 requires about 90 minutes. The Hybridization reaction may be run overnight with the following protocol modifications:

- In segment 5 of the thermal cycler program (Table 27), replace the 65°C Hold step with a 21°C Hold step.
- The hybridized samples may be held at 21°C for up to 16 hours. Begin the capture preparation steps on page 56 on day 2, after the overnight hold.

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

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

3 Transfer the sealed sample plate or strip to the thermal cycler and resume the thermal cycling program (Table 27 on page 52), allowing the cycler to complete Segments 1 and 2 of the program.

Important: The thermal cycler must be paused during Segment 3 to allow additional reagents to be added to the Hybridization wells in step 6 on page 55.

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

4 Prepare a 25% solution of SureSelect RNase Block (1 part RNase Block to 3 parts water) according to Table 28. Prepare the amount required for the number of hybridization reactions in the run, plus excess. Mix well and keep on ice.

Table 28 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 (purple cap)	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 5, below, just before pausing the thermal cycler in Segment 3. Keep the mixture at room temperature briefly until the mixture is added to the DNA samples in step 6 on page 55. Do not keep solutions containing the probe at room temperature for extended periods.

5 Prepare the Probe Hybridization Mix appropriate for your probe design size. Use Table 29 for probes ≥3 Mb or Table 30 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 6.

Table 29 Preparation of Probe Hybridization Mix for **probes ≥3 Mb**

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

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

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

6 Once the thermal cycler starts Segment 3 of the program in Table 27 (1 minute at 65°C), pause the program. With the cycler paused, and while keeping the DNA + Blocker samples in the cycler, transfer 13 µl of the room-temperature Probe Hybridization Mix from step 5 to each sample well.

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

   The hybridization reaction wells now contain approximately 30 µl.

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

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

CAUTION

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

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

Step 3. Prepare streptavidin-coated magnetic beads

The remaining hybridization capture steps use the reagents in Table 31.

NOTE

If performing same-day hybridization and capture, begin the bead preparation steps below approximately one hour after starting hybridization in step 8 on page 55. If performing next-day capture after an overnight hold at 21°C, begin the bead preparation steps below on day 2, just before you are ready to start the capture steps on page 57.

Table 31 Reagents for Capture

Kit Component	Storage Location	Where Used
SureSelect Binding Buffer	SureSelect Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), RT	page 56
SureSelect Wash Buffer 1	SureSelect Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), RT	page 57
SureSelect Wash Buffer 2	SureSelect Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), RT	page 57
Dynabeads MyOne Streptavidin T1	Follow storage recommendations provided by supplier (see Table 4 on page 15)	page 56

1 Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. The magnetic beads settle during storage.

2 For each hybridization sample, add 50 µl of the resuspended beads to wells of a fresh PCR plate or a strip tube.

3 Wash the beads:

   a Add 200 µl of SureSelect Binding Buffer.

   b Mix by pipetting up and down 20 times or cap the wells and vortex at high speed for 5–10 seconds then spin down briefly.

   c Put the plate or strip tube into a magnetic separator device.

   d Wait at least 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 4. Capture the hybridized DNA using streptavidin-coated beads

1 After all streptavidin bead preparation steps are complete, and with the hybridization thermal cycling program in the final hold segment (see Table 27 on page 52), transfer the samples to room temperature.

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

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

3 Incubate the capture plate or strip tube on a 96-well plate mixer, mixing vigorously (at 1400–1800 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, with heated lid ON, held at 70°C until used in step 9.

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

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

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

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

CAUTION

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

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

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

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

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

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

Make sure the beads are in suspension before proceeding.

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

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

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

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

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

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

NOTE

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

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

Step 1. Amplify the captured libraries

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

This step uses the components listed in Table 32. Before you begin, thaw the reagents listed below and keep on ice.

Table 32 Reagents for post-capture PCR amplification

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

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

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

1 Preprogram a thermal cycler (with the heated lid ON) with the program in Table 33. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 33 Post-capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10 to 16 See Table 34 for recommendations based on Probe size	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Table 34 Post-capture PCR cycle number recommendations

Probe Design Size	Cycles
Probes <0.2 Mb	14–16 cycles
Probes 0.2–3 Mb	12–14 cycles
Probes 3–5 Mb	10–12 cycles
Probes >5 Mb (including Human All Exon probes)	10 cycles

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 35, on ice. Mix well on a vortex mixer.

Table 35 Preparation of post-capture PCR Reaction mix

Reagent	Volume for 1 Amplification Reaction	Volume for 6 Amplification Reactions (includes excess)
Nuclease-free water	12.5 µl	87.5 µl
5× Herculase II Reaction Buffer (clear cap)	10 µl	70 µl
Herculase II Fusion DNA Polymerase (red cap)	1 µl	7 µl
100 mM dNTP Mix (green cap)	0.5 µl	3.5 µl
SureSelect Post-Capture Primer Mix (clear cap)	1 µl	7 µl
Total	**25 µl**	**175 µl**

3 Add 25 µl of the PCR reaction mix prepared in Table 35 to each sample well containing 25 µl of bead-bound target-enriched DNA (prepared on page 58 and held on ice).

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

5 Place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 33.

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

The beads can be discarded at this time.

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

In this step, the amplified enriched DNA libraries are purified using AmpPure XP Beads. Critical purification protocol parameters are summarized for experienced users in Table 36.

Table 36 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 1X Low TE Buffer
Amount of eluted sample transferred to fresh well	Approximately 25 µl

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

   Check that the beads are in a homogeneous suspension in the sample wells. Each well should have a uniform color with no layers of beads or clear liquid present.
5. Incubate samples for 5 minutes at room temperature.
6. Put the plate or strip tube on the magnetic stand at room temperature. Wait for the solution to clear (approximately 3 to 5 minutes).
7. While keeping the plate or tubes in the magnetic stand, carefully remove and discard the cleared solution from each well. Do not disturb the beads while removing the solution.
8. Continue to keep the plate or tubes in the magnetic stand while you dispense 200 µl of freshly-prepared 70% ethanol in each sample well.

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

10 Repeat step 8 and step 9 once for a total of two washes. Make sure to remove all of the ethanol at each wash step.

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

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

13 Add 25 µl of 1X Low TE Buffer to each sample well.

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

15 Incubate for 2 minutes at room temperature.

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

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

Step 3. Assess sequencing library DNA quantity and quality

Option 1: Analysis using the Agilent 2100 Bioanalyzer instrument and High Sensitivity DNA Assay

Use the Bioanalyzer High Sensitivity DNA Assay to analyze the amplified captured DNA. Perform the assay according to the High Sensitivity DNA Kit Guide.

1. Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.
2. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µl of each sample for the analysis.
3. Load the prepared chip into the instrument and start the run within five minutes after preparation.
4. Verify that the electropherogram shows the peak of DNA fragment size positioned between 200 and 400 bp. Sample electropherograms are shown in Figure 8 (library prepared from high-quality DNA), Figure 9 (library prepared from medium-quality FFPE DNA), and Figure 10 (library prepared from low-quality FFPE DNA).
5. Measure the concentration of each library by integrating under the entire peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

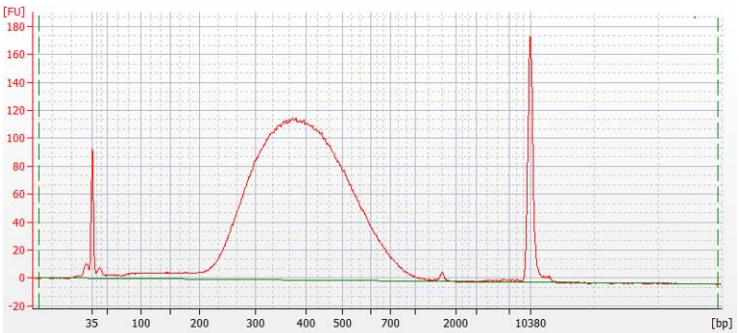


Figure 8 Post-capture library prepared from a high-quality gDNA sample analyzed using a Bioanalyzer system High Sensitivity DNA assay.

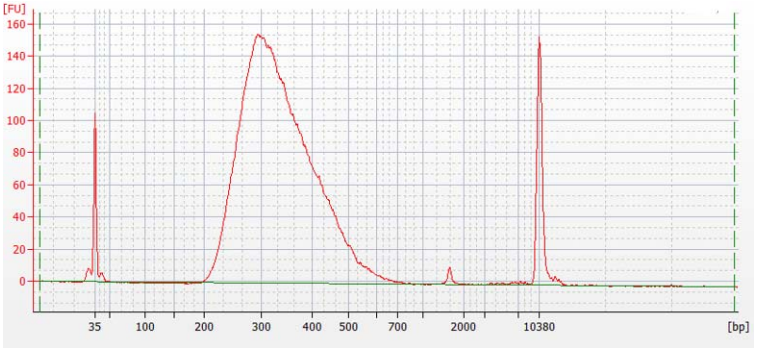


Figure 9 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a Bioanalyzer system High Sensitivity DNA assay.

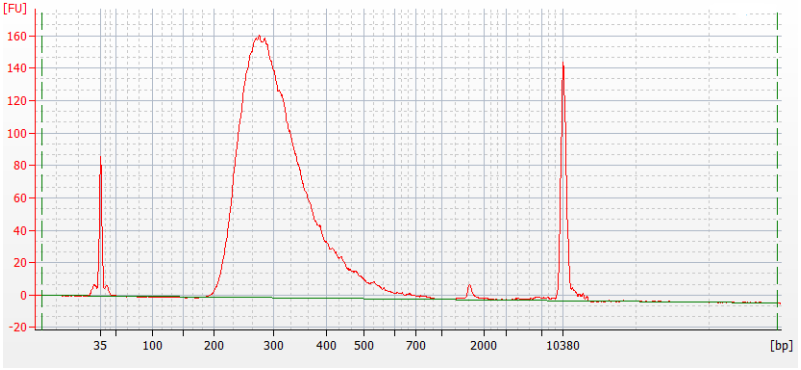


Figure 10 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a Bioanalyzer system High Sensitivity DNA assay.

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

Option 2: Analysis using an Agilent TapeStation and High Sensitivity D1000 ScreenTape

Use a High Sensitivity D1000 ScreenTape and associated reagent kit. For more information to do this step, see the Agilent High Sensitivity D1000 Assay Quick Guide.

1 Prepare the TapeStation samples as instructed in the reagent kit guide. Use 2 µl of each indexed DNA sample diluted with 2 µl of High Sensitivity D1000 sample buffer for the analysis.

CAUTION For accurate quantitation, make sure to thoroughly mix the combined DNA and sample buffer by vortexing the assay plate or tube strip for 1 minute on the IKA MS3 vortex mixer provided with the 4200/4150 TapeStation system before loading the samples.

2 Load the sample plate or tube strips from step 1, the High Sensitivity D1000 ScreenTape, and loading tips into the TapeStation as instructed in the reagent kit guide. Start the run.

3 Verify that the electropherogram shows the peak of DNA fragment size positioned between 200 and 400 bp. Sample electropherograms are shown in Figure 11 (library prepared from high-quality DNA), Figure 12 (library prepared from medium-quality FFPE DNA), and Figure 13 (library prepared from low-quality FFPE DNA).

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

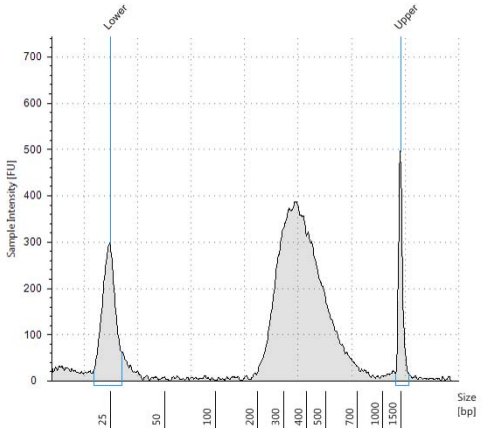


Figure 11 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

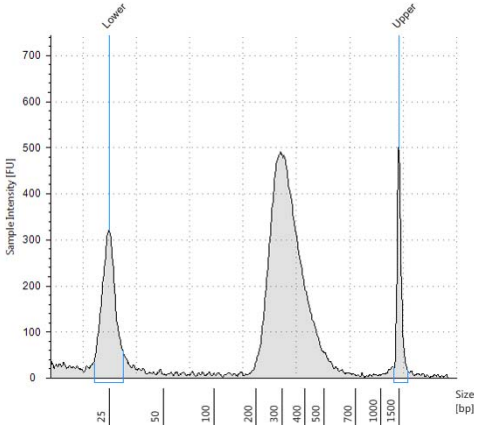


Figure 12 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

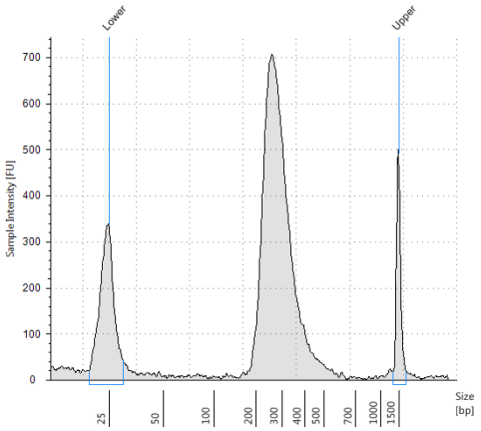


Figure 13 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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

Step 4. Optional: Pool samples for multiplexed sequencing

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

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

If samples will not be further combined in post-capture pools, proceed to page 72.

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

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

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

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

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

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

is the number of samples in final pool, and

C(i) is the initial concentration of sample

Table 37 shows an example of the amount of 4 samples (of different concentrations) and Low TE needed for a final volume of 20 µl at 10 nM DNA.

Table 37 Example of volume calculation for total volume of 20 µl at 10 nM concentration

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

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

Step 5. Prepare sequencing samples

The final SureSelect$^{XT\ HS}$/SureSelect^{XT} Low Input library pool is ready for direct sequencing using standard Illumina paired-end primers and chemistry. Each fragment in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform, as shown in Figure 14.

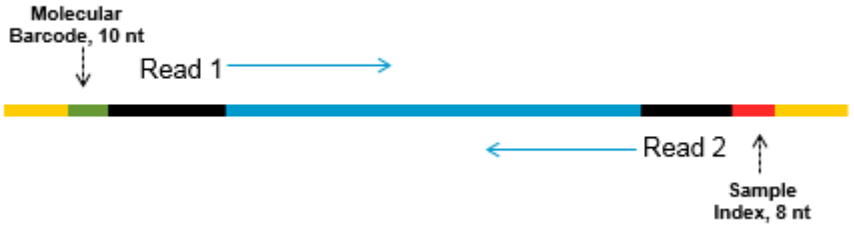


Figure 14 Content of the sequencing library DNA fragments. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the sample index (red), the molecular barcode (MBC; green) and the library bridge PCR primers (yellow). Sequencing of the 10-bp MBC (i5) reads is optional.

Libraries can be sequenced on the Illumina HiSeq, MiSeq, NextSeq, or NovaSeq platform using the run type and chemistry combinations shown in Table 38.

CAUTION Do not use the HiSeq2500 instrument in high-output run mode (v4 chemistry) if your analysis pipeline includes MBC (i5) reads. Poor MBC sequence data quality (lower Q scores, with impacts on coverage and sensitivity of variant calls) has been observed when SureSelect^{XT} Low Input libraries are sequenced on the HiSeq 2500 instrument in this mode. See Table 38 for alternative run mode/chemistry options for the HiSeq2500 platform. Sequencing performance for Read 1, Read 2, and sample-level index (i7) reads is not affected, and this platform/run mode/chemistry may be used for applications that omit MBC analysis.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See Table 38 for kit configurations compatible with the recommended read length.

The optimal seeding concentration for the target-enriched library pools varies according to sequencing platform, run type, and Illumina kit version. See Table 38 for guidelines. Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library pool and on the desired output and data quality.

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

Table 38 Illumina Kit Configuration Selection Guidelines

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

* Do not use HiSeq 2500 High Output (v4 chemistry) runs if your analysis pipeline includes MBC (i5) reads. Reduced MBC sequence quality and lowered Q scores have been observed in sequences obtained from HiSeq 2500 platform runs under these conditions. Sequencing performance for Read 1, Read 2, and sample-level index (i7) reads is not affected.

Step 6. Do the sequencing run and analyze the data

Use the guidelines below for SureSelect$^{XT\ HS}$/SureSelect^{XT} Low Input library sequencing run setup and analysis.

- The sample-level index (i7) requires an 8-bp index read. For complete i7 index sequence information, see Table 49 on page 94.

CAUTION

The 8-bp index sequences in SureSelect XT Low Input Index Primers 1-96 differ from the 8-bp index sequences in index primers A01 through H12 in Agilent's SureSelect XT system.

- Optional use of the degenerate MBC (i5) requires a 10-bp index read.

NOTE

If MBC analysis is not needed, you can modify the steps in this section to omit sequencing and analysis of i5 index reads. Use of the MBCs is recommended for detection of very low allele frequency variants and when the DNA sample is present in limited amounts.

Note that if you are using the SureCall (v4.2) *All-In-One Analysis* workflow for analysis of SureSelect Cancer All-In-One assays, this workflow does not include MBC analysis.

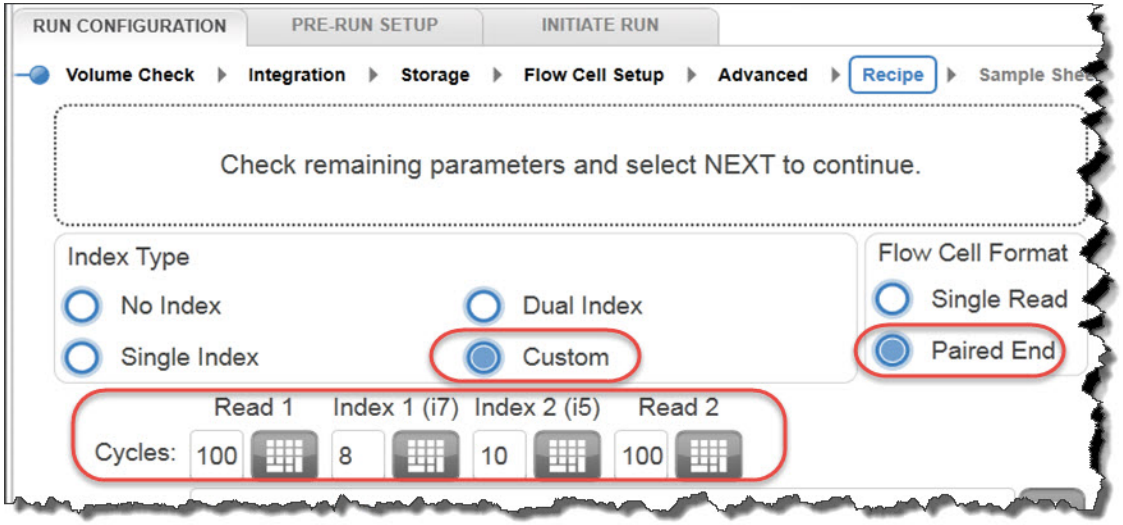
- For the HiSeq, NextSeq, and NovaSeq platforms, set up the run using the instrument's user interface, following the guidelines on page 76.
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed on page 79 to page 82 to generate a custom sample sheet.
- Do not use Illumina's IEM adaptor trimming options. Make sure any IEM adaptor trimming option checkboxes are cleared (deselected) when setting up the sequencing run. Adaptors are trimmed in later processing steps using the Agilent software tools described below to ensure proper processing.
- Demultiplex using the appropriate Illumina software to generate paired-end reads in FASTQ format. If your workflow excludes use of MBCs, you can demultiplex using Illumina's bcl2fastq, BCL Convert or DRAGEN software.

  If your workflow includes use of MBCs, demultiplex using Illumina's bcl2fastq software, using the I2 MBC retrieval steps described below.

- Retrieval of I2 index files containing the MBC (i5) index reads requires offline conversion of .bcl to .fastq files. For information on how to do this step, see page 77 for HiSeq and NextSeq runs and see page 82 for MiSeq runs.
- For human germline DNA variant analysis, you can use Agilent's Alissa Reporter software for the complete FASTQ file to variant discovery process (see page 83 for more information).
- For germline or somatic variant analysis, you can use Agilent's AGeNT software modules to process the library read FASTQ files to analysis-ready BAM files (see page 84 for more information).

HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface. A sample run setup for the HiSeq platform using 100 + 100 bp paired-end sequencing with MBC (i5) data collection is shown below.



If using the NextSeq or NovaSeq platform, locate the same parameters on the *Run Setup* screen, and populate the **Read Length** fields using the **Cycles** settings shown in HiSeq platform example above. In the **Custom Primers** section of the NextSeq or NovaSeq platform *Run Setup* screen, clear (do **not** select) the checkboxes for all primers (*Read 1, Read 2, Index 1* and *Index 2*).

BaseSpace currently does not support the sequencing of MBCs as index reads. Set up NextSeq runs using the stand-alone mode if your analysis pipeline includes MBC analysis.

Retrieve I2 FASTQ files containing MBCs

Retrieval of I2 index files containing the MBC (i5) index reads requires offline conversion of .bcl to .fastq files using one of the two methods below.

Option 1: Use bcl2fastq software with base masking

To generate Index 2 FASTQ files containing the MBCs using the bcl2fastq software, follow Illumina's instructions for use of the software with the following modifications:

1. Use of a sample sheet is mandatory and not optional. Modify the sample sheet to include only the sample index (i7/Index 1) and not the MBC (i5/Index 2) by clearing the contents in the **I5_Index_ID** and **index2** columns.
2. Set ***mask-short-adapter-reads*** to value of 0.
3. Use the following base mask: Y*, I8, Y10, Y* (where * should be replaced with the actual read length, with the value entered matching the read length value in the RunInfo.xml file).

CAUTION

When generating FASTQ files using Illumina's bcl2fastq software, make sure to clear the contents of the **index2** column in the sample sheet as described above. **Do not enter an N_{10} sequence to represent the degenerate MBC**; instead, simply leave the column cells cleared.

The bcl2fastq software does not treat the "N" character as a wildcard when found in sample sheet index sequences, and usage in this context will cause a mismatch for any sequence character other than "N".

Option 2: Use Broad Institute Picard tools

To generate Index 2 FASTQ files containing the P5 MBCs using the Broad Institute Picard tools, complete the following steps:

1. Use tool **ExtractIlluminaBarcodes** to find the barcodes. A sample set of commands is shown below (commands used by your facility may vary).

```
nohup java -jar picard.jar ExtractIlluminaBarcodes
BASECALLS_DIR=<sequencing_run_directory>/Data/Intensities/BaseCalls/
OUTPUT_DIR=<barcode_output_dir_name> LANE=1 READ_STRUCTURE=<read_structure>
BARCODE_FILE=<barcode_file> METRICS_FILE=<metric_file_name>
NUM_PROCESSORS=<n>
```

2 Use tool **IlluminaBaseCallsToFastq** to generate the FASTQ files based on output of step 1. A sample set of commands is shown below (commands used by your facility may vary).

```
nohup java -jar picard.jar IlluminaBasecallsToFastq
BASECALLS_DIR=<sequencing_run_directory>/Data/Intensities/BaseCalls/ LANE=1
BARCODES_DIR=<barcode_output_dir_name> READ_STRUCTURE=<read_structure>
FLOWCELL_BARCODE=<FCID> MACHINE_NAME=<machine_name>
RUN_BARCODE=<run_number> ADAPTERS_TO_CHECK=PAIRED_END

NUM_PROCESSORS=<n> READ_NAME_FORMAT=CASAVA_1_8 COMPRESS_OUTPUTS=true
MULTIPLEX_PARAMS=<multiplex_params_file> IGNORE_UNEXPECTED_BARCODES=true
TMP_DIR=<temp_directory_location>
```

MiSeq platform sequencing run setup guidelines

Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below. Once a Sample Sheet has been generated, index sequences need to be manually changed to the SureSelect Low Input indexes used for each sample. See Table 49 on page 94 for sequences of the SureSelect XT Low Input system indexes.

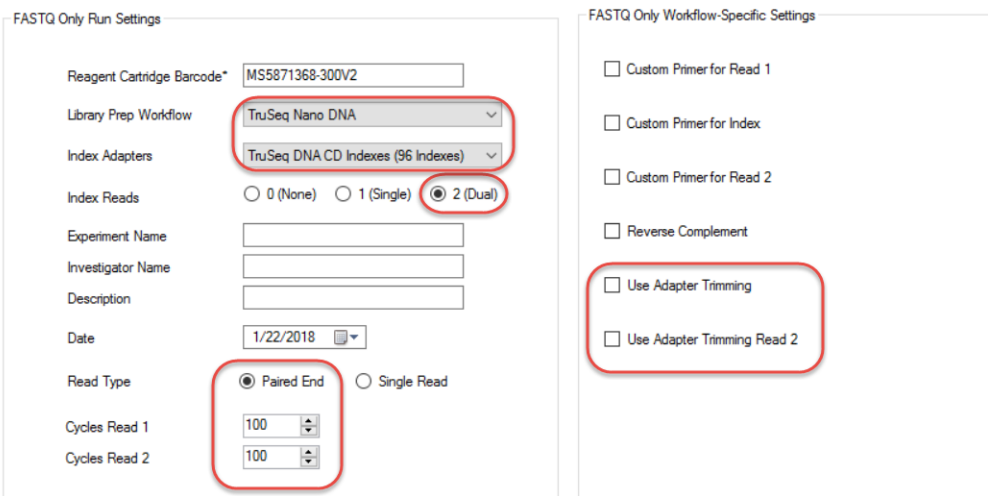
If your workflow excludes use of MBCs, modify the steps in this section to collect a Single Index Read (i7) and omit steps for I2 MBC file retrieval.

Set up a custom Sample Sheet:

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

2 On the **Workflow Parameters** screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA**. In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. Make sure to clear both adaptor-trimming checkboxes under *FASTQ Only Workflow-Specific Settings* (circled below), since these are selected by default.

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



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

Likewise, in the **I5 Sequence** column, assign any of the Illumina i5 indexes, to be corrected to the degenerate MBC at a later stage.

Sample ID*	Sample Name	Plate	Well	Index1 (I7)*	I7 Sequence	Index2 (I5)*	I5 Sequence	Sample Project	Description
1	1	Plate1	A01	D701	ATTACTCG	D501	TATAGCCT		
2	2	Plate1	A02	D702	TCCGGAGA	D501	TATAGCCT		
3	3	Plate1	A03	D703	CGCTCATT	D501	TATAGCCT		
4	4	Plate1	A04	D704	GAGATTCC	D501	TATAGCCT		
5	5	Plate1	A05	D705	ATTCAGAA	D501	TATAGCCT		
6	6	Plate1	A06	D706	GAATTCGT	D501	TATAGCCT		

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

Edit the Sample Sheet to include SureSelect XT Low Input indexes and MBCs

1 Open the Sample Sheet file in a text editor and edit the i7 and i5 index information for each sample in columns 5–8 (highlighted in Figure 15).

- In column 5 under **I7_Index_ID**, enter the name of the SureSelect XT Low Input index assigned to the sample. In column 6 under **index**, enter the corresponding SureSelect XT Low Input Index sequence. See Table 49 on page 94 for index nucleotide sequences.
- In column 7 under **I5_Index_ID**, enter *MBC* for all samples. In column 8 under **index2**, enter text *NNNNNNNNNN* for all samples to represent the degenerate 10-nucleotide MBC tagging each fragment.

NOTE

Enter N_{10} text in the **index2** column only when sample sheets are processed using MiSeq Reporter software adjusted to retrieve I2 FASTQ files containing MBCs, as detailed on page 82. Sample sheets processed offline using Illumina's bcl2fastq software must not contain N_{10} wildcard index sequences. See page 77 for more information.

[Header]									
IEMFileVer	5								
Experimen	XTHS								
Date	1/22/2018								
Workflow	GenerateFASTQ								
Applicatio	FASTQ Only								
Instrumen	MiSeq								
Assay	TruSeq Nano DNA								
Index Ada	TruSeq DNA CD Indexes (96 Indexes)								
Description									
Chemistry	Amplicon								
[Reads]									
100									
100									
[Settings]									
ReverseCo	0								
[Data]									
Sample_ID	Sample_Name	Sample_Plate	Sample_W	Index_Plate_W	I7_Index_ID	index	I5_Index_I	index2	Sampl
XTHS-S1	XTHS-S1	1	A01	A01	A01	GTCTGTCA	MBC	NNNNNNNNNN	XTHS
XTHS-S2	XTHS-S2	1	B01	B01	B01	TGAAGAGA	MBC	NNNNNNNNNN	XTHS

Figure 15 Sample sheet for use with MiSeq platform after MiSeq Reporter reconfiguration

2 Save the edited Sample Sheet in an appropriate file location for use in the MiSeq platform run.

Reconfigure the MiSeq Reporter Software to retrieve I2 FASTQ files

By default, MiSeq Reporter software does not generate FASTQ files for index reads. To generate FASTQ I2 index files containing the MBC reads using MiSeq Reporter, adjust the software settings as described below before the first use of the MiSeq instrument for SureSelect XT Low Input library sequencing. Once changed, this setting is retained for future runs.

To change this setting, open the file **MiSeq Reporter.exe.config**. Under the **<appSettings>** tag, add **<add key="CreateFastqForIndexReads" value="1"/>**. You must restart the instrument for this setting change to take effect.

NOTE

If you are using the same instrument for assays other than SureSelect XT Low Input library sequencing, the configuration file should be edited to **<add key="CreateFastqForIndexReads" value="0"/>** and the instrument should be restarted before running the other assay.

If you are using the MiSeqDx platform, run the instrument in research mode to make changes to MiSeq Reporter settings. If research mode is not available on your instrument, you may need to upgrade the system to include the dual boot configuration to allow settings changes in research mode.

The alternative methods for retrieval of I2 FASTQ files described on page 77 for HiSeq and NextSeq platform runs may also be applied to MiSeq platform runs.

Sequence analysis resources

Guidelines are provided below for typical NGS analysis pipeline steps appropriate for SureSelect XT Low Input DNA library data analysis. Your NGS analysis pipeline may vary. For SureSelect Cancer All-In-One assay sequence analysis guidelines, see the assay Product Overview Guide.

Prior to analysis, use the appropriate Illumina demultiplexing software to generate paired-end reads (see page 74 for guidelines). The demultiplexed FASTQ data needs to be pre-processed to remove sequencing adaptors and utilize the i5 MBC reads (if collected) using one of the tools described below.

Using Agilent's Alissa Reporter software for germline DNA workflows

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

Alissa Reporter is a cloud-based, multi-tenant software as a service (SaaS) product, delivering integrated pre-processing of SureSelect XT Low Input DNA library reads (adaptor trimming, MBC extraction and deduplication) 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 Low Input DNA assay steps prior to Alissa Reporter software analysis are summarized below:

- Alissa Reporter applications are available for germline analysis of human DNA libraries enriched using a pre-designed or custom SureSelect human probe (see page 13). 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.

NOTE

Human All Exon V8+UTR and Human All Exon V8+NCV designs must be imported into Alissa Reporter for use as *Custom*-type applications. Use the *Catalog*-type Alissa Reporter applications, including the *Human All Exon V8 Germline* application, only for the specific probe indicated for the application without any additional design content.

- Analysis of FFPE-derived or other DNA samples for detection of somatic variants is not supported at the time of this publication. Please visit the Alissa Reporter page at www.agilent.com for information on the latest Alissa Reporter software version capabilities.

- For CNV calling a co-analysis strategy is used in which unrelated samples from the same Alissa Reporter run are used to determine the reference signal for the target sample (no specific reference sample is required). 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.
- Maximum file size for uploads is 50GB/file (in total 400GB/sample). A maximum of 768 FASTQ files can be uploaded in a run.
- File sizes>150M reads are randomly subsampled to 150M reads when using the *Human All Exon V7 Germline* or *Human All Exon V8 Germline* application.
- Unmerged and merged FASTQ files are supported. Upload of BAM files or other non-FASTQ file formats is not supported.

Using Agilent's AGeNT software for germline or somatic DNA workflows

Agilent's AGeNT software is a Java-based toolkit used for SureSelect XT Low Input 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 and review AGeNT Best Practices for processing steps suitable for XT HS/Low Input DNA libraries, summarized below.

Prior to variant discovery, the AGeNT Trimmer module is used to pre-process the demultiplexed SureSelect XT Low Input library FASTQ data to trim sequencing adaptors and prepare MBC reads for insertion in the aligned BAM file.

The trimmed reads should be aligned, and MBC tags added to the aligned BAM file 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.

6 Appendix: Using FFPE-derived DNA Samples

This chapter summarizes the protocol modifications to apply to FFPE samples based on the integrity of the FFPE sample DNA.

Protocol modifications for FFPE Samples

Protocol modifications that should be applied to FFPE samples are summarized in Table 39.

Table 39 Summary of protocol modifications for FFPE samples

Workflow Step and page	Parameter	Condition for non-FFPE Samples	Condition for FFPE Samples
gDNA Sample Preparation page 22	Qualification of DNA Integrity	Not required	Required
DNA input for Library Preparation page 22	Input amount and means of quantification	10 ng to 200 ng, quantified by Qubit assay	Based on determined DNA integrity (see Table 8 on page 23 and Table 9 on page 24)
DNA Shearing page 26	Mode of DNA Shearing	2 × 120 seconds	240 seconds (continuous)
Enzymatic Fragmentation of DNA page 28	Duration of 37°C incubation	2 ×100 reads: 15 minutes 2 ×150 reads: 10 minutes	2 ×100 reads: 15 minutes 2 ×150 reads: 15 minutes
Pre-capture PCR page 40	Cycle number	8–11	11–14
Sequencing page 87	Output augmentation	Per project requirements	1× to 10× based on determined DNA integrity (see Table 40 and Table 41 on page 87)

Methods for FFPE Sample Qualification

DNA integrity may be assessed using the Agilent NGS FFPE QC Kit or using the Agilent TapeStation instrument and 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 40. 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 40 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 41. 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 41 Recommended sequencing augmentation for FFPE-derived DNA samples

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

7 Reference

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

Kit Contents

The SureSelect$^{XT\ HS}$/SureSelect^{XT} Low Input Reagent Kits below are used for NGS library preparation using pre-capture pooling. The library preparation reagents supplied with p/n G9916A support preparation of libraries from 96 gDNA samples. The 96 prepared libraries are pooled in sets of 8–16 libraries, depending on the characteristics of the target enrichment design. The hybridization reagents supplied with p/n G9916B support up to 16 hybridization reactions that include 8–16 samples per hybridization.

Table 42 Kits Required for the pre-capture pooling protocol

Ordered Part No.	Component Kit Name	Storage Condition	Component Kit Part No.
G9916A	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), 96 Reactions	–20°C	5500-0140 (see Table 43)
	SureSelect XT Low Input Index Primers 1–96 for ILM (Pre PCR), 96 Reactions	–20°C	5190-6444 (see Table 44)
G9916B	SureSelect Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), 16 Reactions	Room Temperature	5190-9685 (see Table 45)
	SureSelect XT HS Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), 16 Reactions	–20°C	5190-9684 (see Table 46)

The contents of each of the component kits listed in Table 42 are described in the tables below.

Table 43 Content of part number 5500-0140, SureSelect XT HS and XT Low Input Library Preparation Kit (Pre PCR)

Kit Component	Format
End Repair-A Tailing Enzyme Mix	tube with orange cap
End Repair-A Tailing Buffer	bottle
T4 DNA Ligase	tube with blue cap
Ligation Buffer	bottle
Adaptor Oligo Mix	tube with white cap
Forward Primer	tube with brown cap
100 mM dNTP Mix (25 mM each dNTP)	tube with green cap
Herculase II Fusion DNA Polymerase	tube with red cap
5× Herculase II Reaction Buffer	tube with clear cap

Table 44 Content of part number 5190-6444, SureSelect XT Low Input Index Primers for ILM Kits (Pre PCR)

Kit Component	Format
SureSelect XT Low Input Index Primers for ILM (reverse primers containing 8-bp index sequence)	Index Primers 1 through 96, provided in yellow plate (Index Plate 1)*

* See Table 48 on page 93 for a plate map and see Table 49 on page 94 for index sequences.

CAUTION

The SureSelect XT Low Input Index Primers are provided in single-use aliquots. To avoid cross-contamination of libraries, use each well for a single library preparation reaction. Do not re-use any residual volume for subsequent experiments.

Table 45 Content of part number 5190-9685, SureSelect Target Enrichment Kit, ILM Hyb Module Box 1 (Post PCR)

Kit Component	Format
SureSelect Binding Buffer	bottle
SureSelect Wash Buffer 1	bottle
SureSelect Wash Buffer 2	bottle

Table 46 Content of part number 5190-9684, SureSelect XT HS Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR)

Kit Component	Format
SureSelect Fast Hybridization Buffer	bottle
SureSelect XT HS and XT Low Input Blocker Mix	tube with blue cap
SureSelect RNase Block	tube with purple cap
SureSelect Post-Capture Primer Mix	tube with clear cap
100 mM dNTP Mix (25 mM each dNTP)	tube with green cap
Herculase II Fusion DNA Polymerase	tube with red cap
5× Herculase II Reaction Buffer	tube with clear cap

The SureSelect Enzymatic Fragmentation Kit, which may be used for DNA sample fragmentation prior to library preparation, includes the reagents listed in Table 47.

Table 47 Contents of SureSelect Enzymatic Fragmentation Kit (stored at –20°C)

Kit Component	16 Reactions (p/n 5191-4079)	96 Reactions (5191-4080)
SureSelect Fragmentation Enzyme	tube with green cap	tube with green cap
5× SureSelect Fragmentation Buffer	tube with blue cap	tube with blue cap

Table 48 Plate map for SureSelect XT Low Input Index Primers 1-96, provided in yellow plate (Index Plate 1)

	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

Nucleotide Sequences of SureSelect XT Low Input Indexes

Each index is 8 nt in length. See page 74 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 49 SureSelect XT Low Input Indexes 1–96, provided in yellow 96-well plate (Index Plate 1)

Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence
1	A01	GTCTGTCA	25	A04	CCGTGAGA	49	A07	ATGCCTAA	73	A10	ACAGCAGA
2	B01	TGAAGAGA	26	B04	GACTAGTA	50	B07	ATCATTCC	74	B10	AAGAGATC
3	C01	TTCACGCA	27	C04	GATAGACA	51	C07	AACTCACC	75	C10	CAAGACTA
4	D01	AACGTGAT	28	D04	GCTCGGTA	52	D07	AACGCTTA	76	D10	AAGACGGA
5	E01	ACCACTGT	29	E04	GGTGCGAA	53	E07	CAGCGTTA	77	E10	GCCAAGAC
6	F01	ACCTCCAA	30	F04	AACAACCA	54	F07	CTCAATGA	78	F10	CTGTAGCC
7	G01	ATTGAGGA	31	G04	CGGATTGC	55	G07	AATGTTGC	79	G10	CGCTGATC
8	H01	ACACAGAA	32	H04	AGTCACTA	56	H07	CAAGGAGC	80	H10	CAACCACA
9	A02	GCGAGTAA	33	A05	AAACATCG	57	A08	GAATCTGA	81	A11	CCTCCTGA
10	B02	GTCGTAGA	34	B05	ACGTATCA	58	B08	GAGCTGAA	82	B11	TCTTCACA
11	C02	GTGTTCTA	35	C05	CCATCCTC	59	C08	GCCACATA	83	C11	GAACAGGC
12	D02	TATCAGCA	36	D05	GGAGAACA	60	D08	GCTAACGA	84	D11	ATTGGCTC
13	E02	TGGAACAA	37	E05	CGAACTTA	61	E08	GTACGCAA	85	E11	AAGGACAC
14	F02	TGGTGGTA	38	F05	ACAAGCTA	62	F08	TCCGTCTA	86	F11	ACACGACC
15	G02	ACTATGCA	39	G05	CTGAGCCA	63	G08	CAGATCTG	87	G11	ATAGCGAC
16	H02	CCTAATCC	40	H05	ACATTGGC	64	H08	AGTACAAG	88	H11	CCGAAGTA
17	A03	AGCAGGAA	41	A06	CATACCAA	65	A09	AGGCTAAC	89	A12	CCTCTATC
18	B03	AGCCATGC	42	B06	CAATGGAA	66	B09	CGACTGGA	90	B12	AACCGAGA
19	C03	TGGCTTCA	43	C06	ACGCTCGA	67	C09	CACCTTAC	91	C12	GATGAATC
20	D03	CATCAAGT	44	D06	CCAGTTCA	68	D09	CACTTCGA	92	D12	GACAGTGC
21	E03	CTAAGGTC	45	E06	TAGGATGA	69	E09	GAGTTAGC	93	E12	CCGACAAC
22	F03	AGTGGTCA	46	F06	CGCATACA	70	F09	CTGGCATA	94	F12	AGCACCTC
23	G03	AGATCGCA	47	G06	AGAGTCAA	71	G09	AAGGTACA	95	G12	ACAGATTC
24	H03	ATCCTGTA	48	H06	AGATGTAC	72	H09	CGACACAC	96	H12	AATCCGTC

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 FFPE DNA samples is too low for enzymatic fragmentation

- ✔ The standard enzymatic fragmentation protocol requires 10–200 ng input DNA in a volume of 7 µl, and uses a final fragmentation reaction volume of 10 µl. For dilute FFPE samples, enzymatic fragmentation may be performed using the modified protocol below:
  - Bring FFPE samples containing 10–200 ng DNA to 17 µl final volume with 1X Low TE Buffer.
  - Prepare the Fragmentation master mix as directed in Table 13 on page 29.
  - Add 3 µl of the master mix to each 17-µl DNA sample. Mix and spin as directed on page 29.
  - Run the thermal cycling program in Table 11 on page 28 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 33) is kept at room temperature for 30–45 minutes before use.
- ✔ PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the pre-capture PCR cycle number by 1 to 2 cycles. If a high molecular weight peak (>500 bp) is observed in the electropherogram for a sample with low yield, the DNA may be overamplified. Repeat library preparation for the sample, decreasing the pre-capture PCR cycle number by 1 to 3 cycles.
- ✔ DNA isolated from degraded samples, including FFPE tissue samples, may be over-fragmented or have modifications that adversely affect library preparation processes. Use the Agilent NGS FFPE QC Kit to determine the precise quantity of amplifiable DNA in the sample and allow direct normalization of input DNA amount.
- ✔ Performance of the solid-phase reversible immobilization (SPRI) purification step may be poor. Verify the expiration date for the vial of AMPure XP beads used for purification. Adhere to all bead storage and handling conditions recommended by the manufacturer. Ensure that the beads are kept at room temperature for at least 30 minutes before use. Use freshly-prepared 70% ethanol for each SPRI procedure.
- ✔ DNA elution during SPRI purification steps may be incomplete. Ensure that the AMPure XP beads are not overdried just prior to sample elution.

If solids observed in the End Repair-A Tailing Buffer

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

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

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

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

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

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

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

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

✔ The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to the samples analyzed on page 45 to page 48. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield of pre-capture libraries. If excessive adaptor-dimers are observed, verify that the adaptor ligation protocol is being performed as directed on page 36. In particular, ensure that the Ligation master mix is mixed with the sample prior to adding the Adaptor Oligo Mix to the mixture. Do not add the Ligation master mix and the Adaptor Oligo Mix to the sample in a single step.

✔ For whole-genome sequencing (not specifically supported by this protocol), samples with an adaptor-dimer peak must be subjected to an additional round of SPRI-purification. To complete, first dilute the sample to 50 µl with nuclease free water, then follow the SPRI purification procedure on page 42.

If yield of post-capture libraries is low

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

✔ The RNA Probe used for hybridization may have been compromised. Verify the expiration date on the Probe vial or Certificate of Analysis. Adhere to the recommended storage and handling conditions. Ensure that the Probe Hybridization Mix is prepared immediately before use, as directed on page 54, and that solutions containing the Probe are not held at room temperature for extended periods.

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

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

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

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

- SureSelect Wash Buffer 2 is pre-warmed to 70°C (see page 57). 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 58)
- Bead suspensions are mixed thoroughly during washes by pipetting up and down **and** vortexing (see page 58)

✔ Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a vortex and plate spinner or centrifuge in close proximity to thermal cycler to retain the 65°C sample temperature during mixing and transfer steps (step 7 to step 8 on page 55).

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

✔ High AT-dropout may indicate that hybridization conditions are too stringent to obtain the desired level of coverage for AT-rich targets. Repeat target enrichment at lower stringency using a modified thermal cycler program for hybridization, reducing the hybridization temperature in segments 4 and 5 from 65°C to 62.5°C or 60°C (see Table 27 on page 52).

Quick Reference Protocol

An abbreviated summary of the protocol steps is provided below for experienced users. Use the complete protocol on page 22 to page 81 until you are familiar with all of the protocol details such as reagent mixing instructions and instrument settings.

Step	Summary of Conditions
	Library Prep
Prepare, qualify, and fragment DNA samples	Prepare 10–200 ng gDNA (in 50 µl Low TE for Covaris or in 7 µl H_20 for enzymatic fragmentation) For FFPE DNA, qualify integrity and adjust input amount as directed on page 23 and page 24 Mechanically shear DNA using Covaris with shearing conditions on page 26 OR fragment DNA using SureSelect Enzymatic Fragmentation Kit with protocol on page 28 (50 µl final volume)
Prepare Ligation master mix	Per reaction: 23 µl Ligation Buffer + 2 µl T4 DNA Ligase Keep at room temperature 30–45 min before use
Prepare End-Repair/dA-Tailing master mix	Per reaction: 16 µl End Repair-A Tailing Buffer + 4 µl End Repair-A Tailing Enzyme Mix Keep on ice
End-Repair and dA-Tail the sheared DNA	50 µl fragmented DNA sample + 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 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
Prepare PCR master mix	Per reaction: 10 µl 5× Herculase II Reaction Buffer + 0.5 µl 100 mM dNTP Mix + 2 µl Forward Primer + 1 µl Herculase II Fusion DNA Polymerase Keep on ice
Amplify the purified DNA	34.5 µl purified DNA + 13.5 µl PCR master mix + 2 µl assigned SureSelect XT Low Input Index Primer Amplify in thermal cycler using program on page 40
Purify amplified DNA	50 µl amplified DNA + 50 µl AMPure XP bead suspension Elute DNA in 15 µl nuclease-free H_2O
Quantify and qualify DNA	Analyze 1 µl using Agilent 2100 Bioanalyzer or 4200 TapeStation instrument

Step	Summary of Conditions
	Hybridization/Capture
Prep DNA in hyb plate	Pool equal amounts of indexed libraries for total of 1.5 or 3 µg library DNA per pool (see page 50). Adjust volume to 12 µl with nuclease-free H_2O (and using vacuum concentrator when needed).
Program thermal cycler	Input thermal cycler program on page 52 and pause program
Run pre-hybridization blocking protocol	12 µl library DNA pool + 5 µl SureSelect XT HS and XT Low Input Blocker Mix Run paused thermal cycler program segments 1 through 3; start new pause during segment 3 (1 min @ 65°C)
Prepare Probe Hyb Mix	Prepare 25% RNAse Block dilution, then prepare appropriate mixture below: **Probes ≥3 Mb:** 2 µl 25% RNase Block + 5 µl Probe + 6 µl SureSelect Fast Hybridization Buffer **Probes <3 Mb:** 2 µl 25% RNase Block + 2 µl Probe + 3 µl nuclease-free H_2O + 6 µl SureSelect Fast Hybridization Buffer
Run the hybridization	With cycler paused and samples retained in cycler, add 13 µl Probe Hyb Mix to wells Resume the thermal cycler program, completing the remaining hybridization segment and 65°C or 21°C hold segment
Prepare streptavidin beads	Wash 50 µl Dynabeads MyOne Streptavidin T1 beads 3× in 200 µl SureSelect Binding Buffer
Capture hybridized libraries	Add hybridized samples (~30 µl) to washed streptavidin beads (200 µl) Incubate 30 min at RT with vigorous shaking (1400-1800 rpm) During incubation, pre-warm 6 × 200 µl aliquots per sample of SureSelect Wash Buffer 2 to 70°C
Wash captured libraries	Collect streptavidin beads with magnetic stand, discard supernatant Wash beads 1× with 200 µl SureSelect Wash Buffer 1 at RT Wash beads 6× with 200 µl pre-warmed SureSelect Wash Buffer 2 (5 minutes at 70°C per wash) Resuspend washed beads in 25 µl nuclease-free H_2O
	Post-capture amplification
Prepare PCR master mix	Per reaction: 12.5 µl nuclease-free H_2O+ 10 µl 5× Herculase II Reaction Buffer + 0.5 µl 100 mM dNTP Mix + 1 µl SureSelect Post-Capture Primer Mix + 1 µl Herculase II Fusion DNA Polymerase Keep on ice
Amplify the bead-bound captured libraries	25 µl DNA bead suspension+ 25 µl PCR master mix Amplify in thermal cycler using conditions on page 61
Purify amplified DNA	Remove streptavidin beads using magnetic stand; retain supernatant 50 µl amplified DNA + 50 µl AMPure XP bead suspension Elute DNA in 25 µl Low TE
Quantify and qualify DNA	Analyze 1 µl using Agilent 2100 Bioanalyzer or 4200 TapeStation instrument

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In This Book

This guide contains information to prepare pre-capture pooled, target-enriched NGS libraries using the SureSelect$^{XT\ HS}$ / SureSelect^{XT} Low Input target enrichment system.

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