Preparation of Dual Indexed Libraries using SureSelect^{XT} Low Input Target Enrichment System

For Illumina Multiplexed Sequencing Platforms

Protocol

Version D0, November 2020

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 dual-indexed, target-enriched Illumina paired-end sequencing libraries using SureSelect^{XT} Low Input Reagent Kits and Dual Index P5 Indexed Adaptors 1-96 for ILM.

1 Before You Begin

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

2 Preparation and Fragmentation of Input DNA

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

3 Library Preparation

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

4 Hybridization and Capture

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

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

6 Appendix: Using FFPE-derived DNA Samples

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

7 Reference

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

What's New in Version D0

- Addition of optional Enzymatic DNA Fragmentation protocol (see page 29 to page 30 for the protocol; also see Table 37 on page 84, Table 41 on page 88, and Troubleshooting on page 96)
- New chapter "Preparation and Fragmentation of Input DNA" starting on page 21 (includes input DNA preparation and mechanical shearing information previously found in "Sample Preparation" chapter)
- Addition of hybridization temperature considerations for probes designed for use with the SureSelect XT system (see footnote to Table 24 on page 53)
- Minor updates to instructions in the "Hybridization and Capture" chapter, including *Note* on page 53, step 3 on page 53, step 5 on page 54, and step 1 on page 58
- Updates to downstream sequencing platform and kit support information (Table 35 on page 75, Table 50 on page 94 and Table 51 on page 95)
- Addition of small volume spectrophotometer to Table 5 on page 16
- Update to description of flat strip caps in Table 7 on page 19

What's New in Version C0

- Support for revised SureSelect custom probe products, produced using an updated manufacturing process beginning August, 2020 (see Table 3 on page 14). Custom probes produced using the legacy manufacturing process are also fully supported by the protocols in this document. Probe nomenclature was updated throughout document.
- Support for separately-purchased SureSelect XT Low Input Reagent Kit G9703A and Probe (see Table 2 on page 13 and Table 3 on page 14). Bundled SureSelect XT Low Input Reagent Kit + Probe products (Agilent p/n G9707A-S) are also supported by the protocols in this

document; see page 91 for information on available Reagent Kit + Probe bundles.

- Reorganization of tables in section "Materials Required" on page 13 to highlight requirements for specific workflow options.
- Updates to thermal cycler recommendations and usage instructions (see *Caution* and Table 5 on page 16 and example usage instructions in step 2 on page 34).
- Updates to *Materials Required* including updated ordering information for Dynabeads MyOne Streptavidin T1 beads and AMPure XP Kits (Table 4 on page 15) and for Eppendorf ThermoMixer C and Qubit Fluorometer (Table 5 on page 16).
- Updates to "Optional Materials" on page 19, including removal of ethylene glycol supplier information (see page 26 for related update to DNA shearing set up instructions).
- Updates to Agilent TapeStation 4200/4150 ordering and sample mixing information (see Table 5 on page 16 and see *Caution* on page 47 and page 69).
- Support for 5200 Fragment Analyzer (see footnote to Table 5 on page 16).
- Support for optional overnight hybridization (see *Note* on page 53).
- Update to Table 14 on page 32 to include thawing of P5 Indexed Adaptors in advance of use.
- Formulation of 1X Low TE Buffer added to step 3 on page 22.
- Update to instructions in step 3 on page 57 to include brief spin when washes mixed by vortexing.
- Updates to Technical Support contact information (see page 2).
- Updates to *Notice to Purchaser* (see page 2).

Content

1 Before You Begin

Make sure you have the most current protocol. Go to genomics.agilent.com and search for G9703-90050.

To prepare dual-indexed libraries for Agilent SureSelect Cancer All-In-One assays, use the protocols detailed in this publication, while implementing the considerations provided in the SureSelect Cancer All-In-One Target Enrichment Product Overview Guide (publication G9702-90100).

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. Make sure to use P5 Indexed Adaptors instead of the Adaptor Oligo Mix and 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 dual indexing SureSelect^{XT} Low Input with Dual Indexing workflow is summarized in Figure 1. The estimated time requirements for each step are summarized in Table 1.

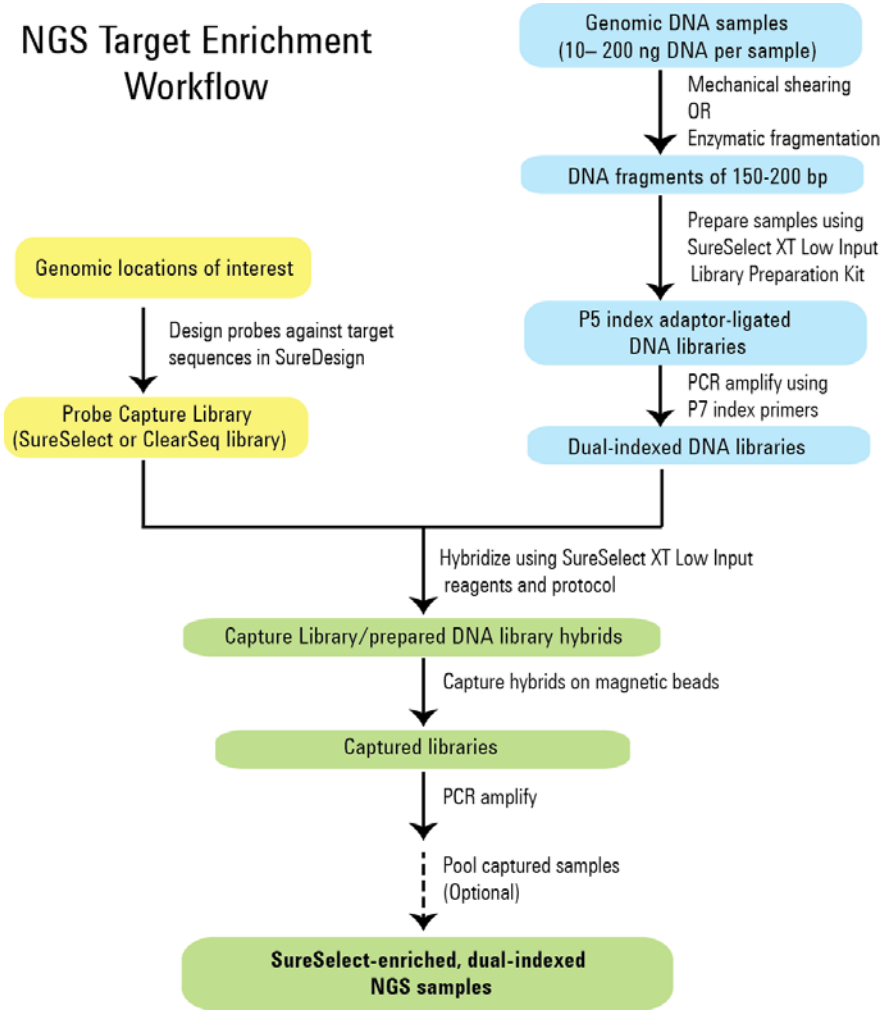


Figure 1 Overall dual-indexed, target-enriched sequencing sample preparation workflow.

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

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

Procedural Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Use best-practices to prevent PCR product contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow.
  2. Maintain clean work areas. Clean pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution.
  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. Domed cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler and from other procedural steps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during thermal cycler incubation steps.
- In general, follow Biosafety Level 1 (BSL1) safety rules.
- Possible stopping points, where samples may be stored at –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.

Safety Notes

CAUTION

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

Materials Required

Materials required to complete the SureSelect^{XT} Low Input Dual Indexing 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

Materials listed in Table 2, plus a compatible target enrichment Probe (selected from Table 3 on page 14) are required for all Dual Indexing workflows. Refer to Table 4 through Table 6 for additional materials needed to complete the protocols according to your DNA sample type/fragmentation method.

Table 2 SureSelect Reagents for Dual Indexing Workflows

Description	96 Reaction Kit Part Number*
SureSelect XT Low Input Reagent Kit with Index Primers 1–96 for Illumina (ILM) platform†	G9703A
SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM	5191-4056

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

† Compatible with HiSeq, MiSeq, NextSeq 500, and NovaSeq 6000 platforms.

Table 3 Compatible Probes*

Probe Capture Library	Part Number (96 Reactions)
Pre-designed Probes	
SSel XT HS and XT Low Input Human All Exon V7	5191-4029
SureSelect XT Human All Exon V6	5190-8864
SureSelect XT Human All Exon V6 + UTRs	5190-8882
SureSelect XT Clinical Research Exome V2	5190-9492
ClearSeq Comprehensive Cancer XT	5190-8012
Custom Probes†	
SureSelect Custom Tier1 1–499 kb	Please visit the SureDesign website to design Custom SureSelect probes and obtain ordering information. Contact the SureSelect support team (see page 2) or your local representative if you need assistance. Custom probes are also available in a 480 Reaction package size.
SureSelect Custom Tier2 0.5 –2.9 Mb	
SureSelect Custom Tier3 3 –5.9 Mb	
SureSelect Custom Tier4 6 –11.9 Mb	
SureSelect Custom Tier5 12–24 Mb	
Pre-designed Probes customized with additional *Plus* custom content	
SSel XT HS and XT Low Input Human All Exon V7 Plus 1	Please visit the SureDesign website to design the customized *Plus* content and obtain ordering information. Contact the SureSelect support team (see page 2) or your local representative if you need assistance.
SSel XT HS and XT Low Input Human All Exon V7 Plus 2	
SureSelect XT Human All Exon V6 Plus 1	
SureSelect XT Human All Exon V6 Plus 2	
SureSelect XT Clinical Research Exome V2 Plus 1	
SureSelect XT Clinical Research Exome V2 Plus 2	

* Protocols in this document are also compatible with bundled SureSelect XT Low Input Reagent Kits + Target Enrichment Probes, ordered using p/n G9707A-S. See page 91 for more information.

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

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

Description	Vendor and part number
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, domed	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
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 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

* 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 71)
8× flat strip caps	Consult the thermal cycler manufacturer's recommendations	Sealing wells for protocol steps outside of hybridization/capture*
MicroAmp Clear Adhesive Film	Thermo Fisher Scientific p/n 4311971	Improved sealing for flat strip caps*
PlateLoc Thermal Microplate Sealer with Small Hotplate and Peelable Aluminum Seal for PlateLoc Sealer	Please contact the SureSelect support team (see page 2) or your local representative for ordering information	Sealing wells for protocol steps performed inside or outside of the thermal cycler

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

2 Preparation and Fragmentation of Input DNA

This chapter describes the steps to prepare, quantify, qualify, and fragment input DNA samples prior to SureSelect XT 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 83.

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.

Step 1. Prepare and analyze quality of genomic DNA samples

NOTE

If you are preparing DNA samples for an Agilent SureSelect Cancer All-In-One assay, use the following modifications to the gDNA sample preparation instructions in this section:

- Where required for your experimental design, make sure to prepare reference DNA sample(s) alongside your experimental samples
- Use at least 50 ng input gDNA for best results

See publication G9702-90100 for more information.

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 26.

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.

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 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 96 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 below 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

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 sample preparation protocol is used to prepare DNA libraries for sequencing using the Illumina paired-read platform. For each sample to be sequenced, an individual dual-indexed library is prepared. For an overview of the SureSelect^{XT} Low Input target enrichment workflow, see Figure 1 on page 10.

The NGS library preparation protocol that begins here is used for fragmented DNA samples produced by mechanical shearing (as detailed on page 26 to page 28) or produced by enzymatic fragmentation (as detailed on page 29 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 in Table 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 38. *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 34
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 34
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
P5 Indexed Adaptors (green plate)	SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM, –20°C	Thaw on ice then keep on ice	Vortexing	page 37

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 a flat-top vortex mixer when vortexing strip tubes or plates throughout the protocol. When 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 8 reactions* (includes excess)	Volume for 24 reactions* (includes excess)
Ligation Buffer (bottle)	23 µl	207 µl	575 µl
T4 DNA Ligase (blue cap)	2 µl	18 µl	50 µl
Total	**25 µl**	**225 µl**	**625 µl**

* The minimum supported run size for 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

* When setting up the thermal cycling program, use a reaction volume setting of 70 µL.

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

5 Add 20 µl of the End Repair/dA-Tailing master mix to each sample well containing approximately 50 µl fragmented DNA. Mix by pipetting up and down 15–20 times using a pipette set to 60 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 P5-indexed adaptor

Before completing this step, assign a dual index pair to each sample. Use indexing pairs made up of the SureSelect XT Low Input Dual Index P5 Indexed Adaptor (green plate) and the SureSelect XT Low Input Index Primer (providing the P7 index; yellow plate) from the same well position of each index plate. Use a different pair of indexes for each sample to be sequenced in the same lane.

The P5 Indexed Adaptors are ligated to DNA libraries in step 4 below. (See Table 50 on page 94 or Table 51 on page 95 for sequences of the 8-bp P5 index portion of the adaptors.) The P7 indexes are introduced during PCR amplification on page 42. (See Table 49 on page 93 for sequences of the 8-bp P7 index portion of the primers.)

1 Once the thermal cycler program for End Repair/dA-Tailing 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 85 µl or cap the wells and vortex at high speed for 5–10 seconds. Briefly spin the samples.

4 Add 5 µl of the appropriate SureSelect XT Low Input Dual Index P5 Indexed Adaptor (green plate) to each sample. Use a different adaptor for each sample. Mix by pipetting up and down 15–20 times using a pipette set to 85 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 P5 Indexed Adaptor 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.

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

1. Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use. *Do not freeze the beads at any time.*
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 19. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 19 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 42
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 42
100 mM dNTP Mix (green cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	page 42
Forward Primer (brown cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	page 42
SureSelect XT Low Input Index Primers (yellow plate)	SureSelect XT Low Input Index Primers for ILM (Pre PCR), –20°C	Vortexing	page 42

1 Preprogram a thermal cycler (with the heated lid ON) with the program in Table 20. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 20 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 21)	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 21 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.

2 Prepare the appropriate volume of pre-capture PCR reaction mix, as described in Table 22, on ice. Mix well on a vortex mixer.

Table 22 Preparation of Pre-Capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
5× Herculase II Reaction Buffer (clear cap)	10 µl	90 µl	250 µl
100 mM dNTP Mix (green cap)	0.5 µl	4.5 µl	12.5 µl
Forward Primer (brown cap)	2 µl	18 µl	50 µl
Herculase II Fusion DNA Polymerase (red cap)	1 µl	9 µl	25 µl
Total	**13.5 µl**	**121.5 µl**	**337.5 µl**

3 Add 13.5 µl of the PCR reaction mixture prepared in Table 22 to each purified DNA library sample (34.5 µl) in the PCR plate wells.

4 Add 2 µl of the appropriate SureSelect XT Low Input Index Primer (yellow plate; containing P7 index) to each reaction. Use the same P7 index number/plate position as the index number/plate position of the specific P5 Indexed Adaptor ligated to the library in step 4 on page 37.

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.

5 Before adding the samples to the thermal cycler, resume the program in Table 20 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

1. Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use. *Do not freeze the beads at any time.*
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 43.

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 46. See Troubleshooting information on page 98 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.

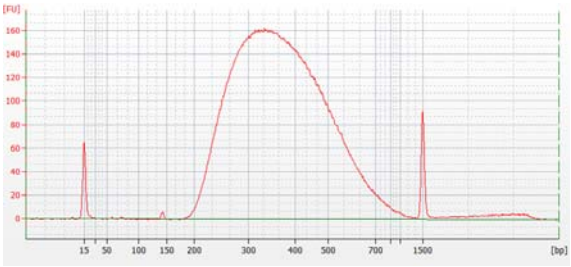


Figure 2 Pre-capture library prepared from a high-quality gDNA sample analyzed using a DNA 1000 Bioanalyzer assay.

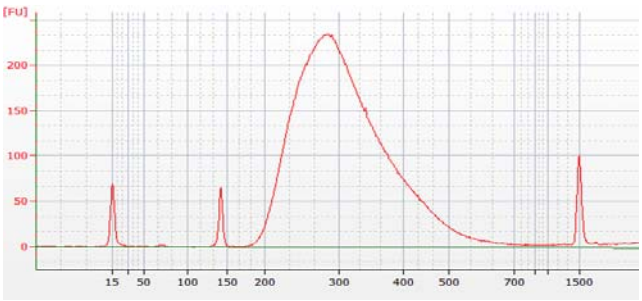


Figure 3 Pre-capture library prepared from a typical FFPE gDNA sample analyzed using a DNA 1000 Bioanalyzer assay.

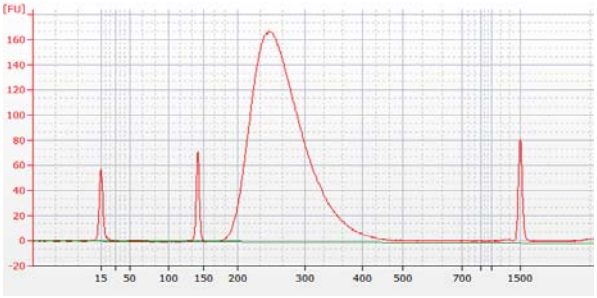


Figure 4 Pre-capture library prepared from a low-quality FFPE gDNA sample analyzed using a DNA 1000 Bioanalyzer assay.

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 instrument user manual. 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 instrument user manual. 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 48 to page 49. See Troubleshooting information on page 98 for additional considerations.

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

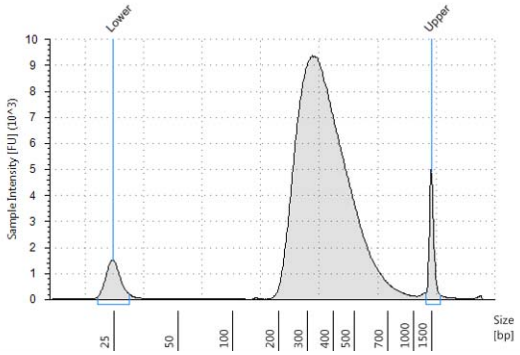


Figure 5 Pre-capture library prepared from a high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

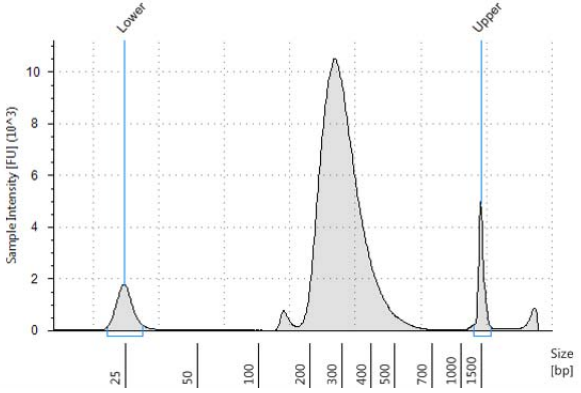


Figure 6 Pre-capture library prepared from a typical FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

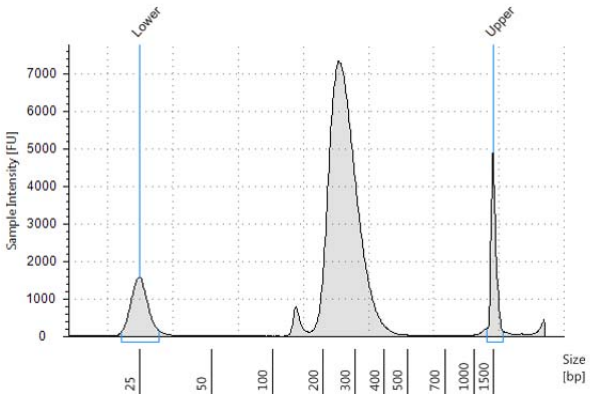


Figure 7 Pre-capture library prepared from a low-quality FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

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 hybridize the prepared gDNA libraries with a target-specific Probe Capture Library. After hybridization, the targeted molecules are captured on streptavidin beads. Each DNA library sample must be hybridized and captured individually.

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 53.

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

Step 1. Hybridize DNA samples to the probe

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

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

This step uses the components listed in Table 23. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 23 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 and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	page 54
SureSelect RNase Block (purple cap)	SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	page 55
SureSelect Fast Hybridization Buffer (bottle)	SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw and keep at Room Temperature	page 55
Probe Capture Library	–80°C	Thaw on ice	page 55

1 Preprogram a thermal cycler (with the heated lid ON) with the program in Table 24. Immediately pause the program, and keep paused until samples are loaded in step 4.

Table 24 Pre-programmed thermal cycler program for Hybridization*

Segment Number	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	65°C	10 minutes
3	1	65°C	1 minute
4	60	65°C†	1 minute
		37°C	3 seconds
5	1	65°C†	Hold

* When setting up the thermal cycling program, use a reaction volume setting of 30 µl (final volume of hybridization reactions during cycling in Segment 4).

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

NOTE

The Hybridization thermal cycling program in Table 24 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 24), 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. Complete the streptavidin bead preparation steps on page 57 just before you are ready to start the capture steps on page 58. Move the hybridized samples to room temperature just before combining samples with the washed streptavidin beads.

2 Place 500–1000 ng of each prepared gDNA library sample into the hybridization plate or strip tube wells and then bring the final volume in each well to 12 µl using nuclease-free water. Use the maximum possible amount of each prepped DNA, within the 500–1000 ng range.

3 To each DNA library sample well, 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.

4 Transfer the sealed sample plates or strips to the thermal cycler and resume the thermal cycling program set up on page 53 and shown in Table 25 below.

Important: Notice that the thermal cycler must be paused during Segment 3 (see Table 25) to allow additional reagents to be added to the Hybridization wells, as described in step 7 on page 56.

During Segments 1 and 2 of the thermal cycling program below, begin preparing the additional reagents as described in step 5 and step 6 on page 55. If needed, you can finish these preparation steps after pausing the thermal cycler in Segment 3.

Table 25 Thermal cycler program for Hybridization with required pause

Segment Number	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)**
4	60	65°C	1 minute
		37°C	3 seconds
5	1	65°C	Hold*

* Begin the capture steps on page 57 as soon as the thermal cycler starts the 65°C Hold segment.

5 Prepare a 25% solution of SureSelect RNase Block (1 part RNase Block to 3 parts water) according to Table 26. Prepare the amount required for the number of hybridization reactions in the run, plus excess. Mix well and keep on ice.

Table 26 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 6, below, just before pausing the thermal cycler in Segment 3 as described on page 54. Keep the mixture at room temperature briefly until the mixture is added to the DNA samples in step 7 on page 56. Do not keep solutions containing the Probe at room temperature for extended periods.

6 Prepare the Capture Library Hybridization Mix appropriate for your probe design size. Use Table 27 for probes ≥3 Mb or Table 28 for probes <3 Mb.

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

Table 27 Preparation of Capture Library Hybridization Mix for **probes ≥3 Mb**

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

Table 28 Preparation of Capture Library Hybridization Mix for **probes <3 Mb**

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

7 Once the thermal cycler starts Segment 3 of the program in Table 25 (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 Capture Library Hybridization Mix from step 6 to each sample well.

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

   The hybridization reaction wells now contain approximately 30 µl.

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

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

CAUTION

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

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

Step 2. Prepare streptavidin-coated magnetic beads

The remaining hybridization capture steps use the components listed in Table 29.

Begin the bead preparation steps described below approximately one hour after starting hybridization in step 9 on page 56.

Table 29 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 57
SureSelect Wash Buffer 1	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	page 58
SureSelect Wash Buffer 2	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	page 58
Dynabeads MyOne Streptavidin T1	Follow storage recommendations provided by supplier (see Table 4 on page 15)	page 57

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 3. Capture the hybridized DNA using streptavidin-coated beads

1. After all streptavidin bead preparation steps are complete, and once the hybridization thermal cycling program reaches the 65°C hold step (see Table 25 on page 54), 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 64.

NOTE

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

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

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 30. Before you begin, thaw the reagents listed below and keep on ice.

Table 30 Reagents for post-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS and XT Low Input Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Pipette up and down 15–20 times	page 64
5× Herculase II Reaction Buffer (clear cap)	SureSelect XT HS and XT Low Input Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Vortexing	page 64
100 mM dNTP Mix (green cap)	SureSelect XT HS and XT Low Input Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Vortexing	page 64
SureSelect Post-Capture Primer Mix (clear cap)	SureSelect XT HS and XT Low Input Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Vortexing	page 64

Prepare one amplification reaction for each DNA library.

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

1 Preprogram a thermal cycler (with the heated lid ON) with the program in Table 31. Immediately pause the program, and keep paused until samples are loaded in step 5.

Table 31 Post-capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	9 to 14 See Table 32 for hybridization probe design size-based cycle number recommendations	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Table 32 Post-capture PCR cycle number recommendations

Probe Size/Description	Cycles
Probes <0.2 Mb	14 cycles
Probes 0.2–3 Mb (includes SSel XT HS and XT Low Input ClearSeq Comp Cancer)	12 cycles
Probes 3–5 Mb	10 cycles
Probes >5 Mb (includes Human All Exon V6, Human All Exon V7 and Clinical Research Exome V2)	9 cycles

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 33, on ice. Mix well on a vortex mixer.

Table 33 Preparation of post-capture PCR Reaction mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
Nuclease-free water	12.5 µl	112.5 µl	312.5 µl
5× Herculase II Reaction Buffer (clear cap)	10 µl	90 µl	250 µl
Herculase II Fusion DNA Polymerase (red cap)	1 µl	9 µl	25 µl
100 mM dNTP Mix (green cap)	0.5 µl	4.5 µl	12.5 µl
SureSelect Post-Capture Primer Mix (clear cap)	1 µl	9 µl	25 µl
Total	**25 µl**	**225 µl**	**625 µl**

3 Add 25 µl of the PCR reaction mix prepared in Table 33 to each sample well containing 25 µl of bead-bound target-enriched DNA (prepared on page 59 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 31.

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

The beads can be discarded at this time.

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

1. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
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 nuclease-free water 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 indexed 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.

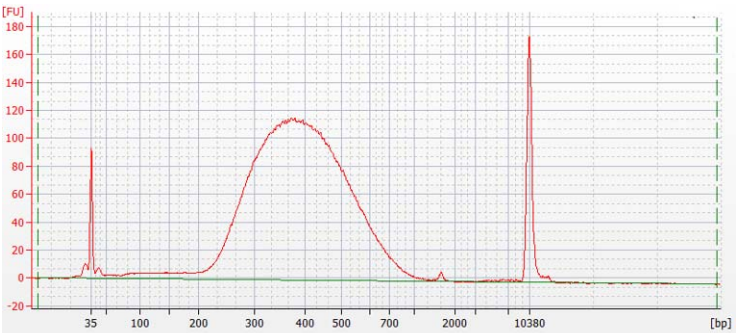


Figure 8 Post-capture library prepared from a high-quality gDNA sample analyzed using a Bioanalyzer system High Sensitivity DNA assay.

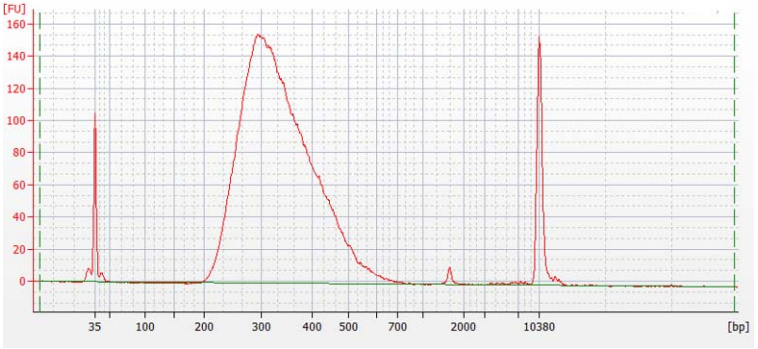


Figure 9 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a Bioanalyzer system High Sensitivity DNA assay.

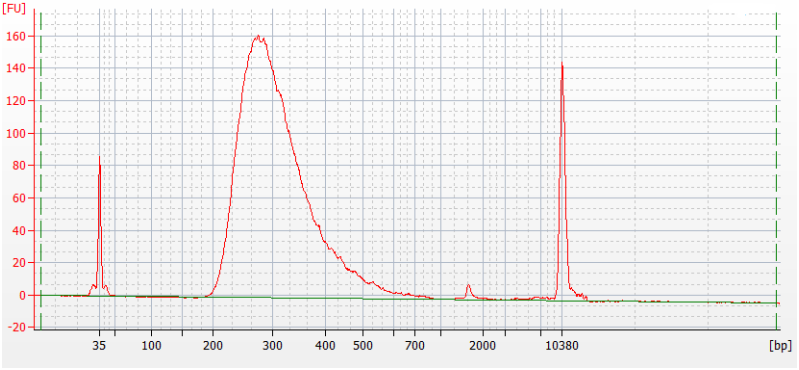


Figure 10 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a Bioanalyzer system High Sensitivity DNA assay.

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 in the reagent kit guide. Use 2 µl of each dual-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 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).

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

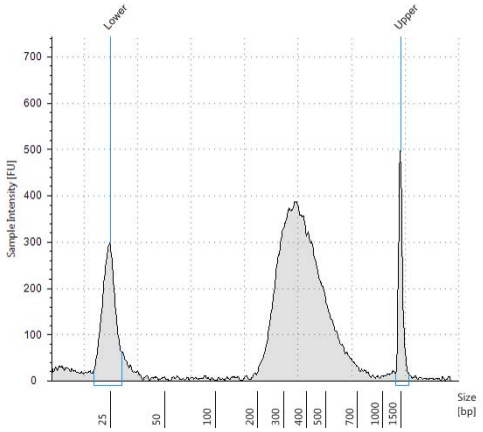


Figure 11 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

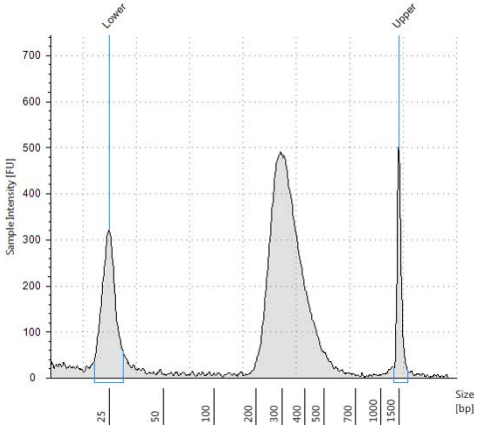


Figure 12 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

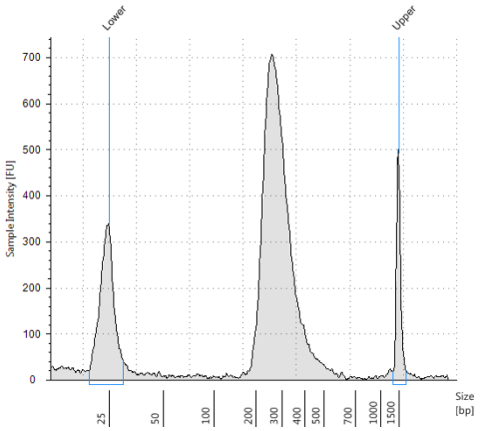


Figure 13 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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. Pool samples for multiplexed sequencing

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

Combine the libraries such that each dual-indexed 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.

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

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

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

is the number of dual-indexed samples, and

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

Table 34 shows an example of the amount of 4 dual-indexed samples (of different concentrations) and Low TE needed for a final volume of 20 µl at 10 nM DNA.

Table 34 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} 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.

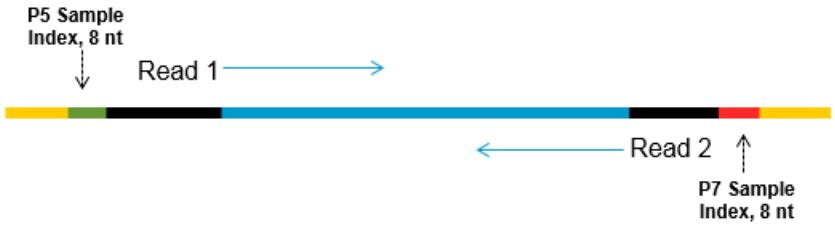


Figure 14 Content of SureSelect XT Low Input dual-indexed sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the P5 and P7 sample indexes (green and red), and the library bridge PCR primers (yellow).

Libraries can be sequenced on the Illumina HiSeq, MiSeq, NextSeq, or NovaSeq platform using the run type and chemistry combinations shown in Table 35.

CAUTION Reduced P5 sample index quality has been observed when SureSelect^{XT} Low Input libraries are sequenced on the HiSeq2500 instrument in high-output run mode (v4 chemistry). Lower Q scores have been shown to impact coverage and sensitivity of variant calls, especially for aberrations present at less than 10% frequency.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See Table 35 for kit configurations compatible with the recommended read length.

The optimal seeding concentration for SureSelect^{XT} Low Input target-enriched libraries varies according to sequencing platform, run type, and Illumina kit version. See Table 35 for guidelines. Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in Table 35.

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

Table 35 Illumina Kit Configuration Selection Guidelines

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

* Reduced i5 index sequence quality and lowered Q scores have been observed in sequences obtained from HiSeq 2500 High Output (v4 chemistry) runs.

NOTE For All-In-One assays that include translocation detection, Agilent strongly recommends using paired-end sequencing read length of at least 2 × 100 bp and preferably 2 × 150 bp.

Step 6. Do the sequencing run and analyze the data

Use the guidelines below for 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 93.

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.

- The sample-level index (i5) requires an 8-bp index read. For complete i5 index sequence information, see Table 50 on page 94 and Table 51 on page 95.
- For the HiSeq, NextSeq, and NovaSeq platforms, set up the run using the instrument's user interface, following the guidelines on page 77.
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed on page 77 to page 80 to generate a custom sample sheet.
- Demultiplex using Illumina's bcl2fastq software to generate paired end reads based on the dual indexes and removing sequences with incorrectly paired P5 and P7 indexes.
- Before aligning reads to the reference genome, trim the reads from Illumina adaptor sequences. See page 81 for information on Agilent's SureCall data analysis software, which may be used for this task.

HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface, using the settings shown in Table 36. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface and enter the **Cycles** settings in Table 36.

For the NextSeq or NovaSeq platform, open the *Run Setup* screen of the instrument control software interface and enter the **Read Length** settings in Table 36. In the **Custom Primers** section, clear (do **not** select) the checkboxes for all primers (*Read 1, Read 2, Index 1* and *Index 2*).

Table 36 Run settings

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

MiSeq platform sequencing run setup guidelines

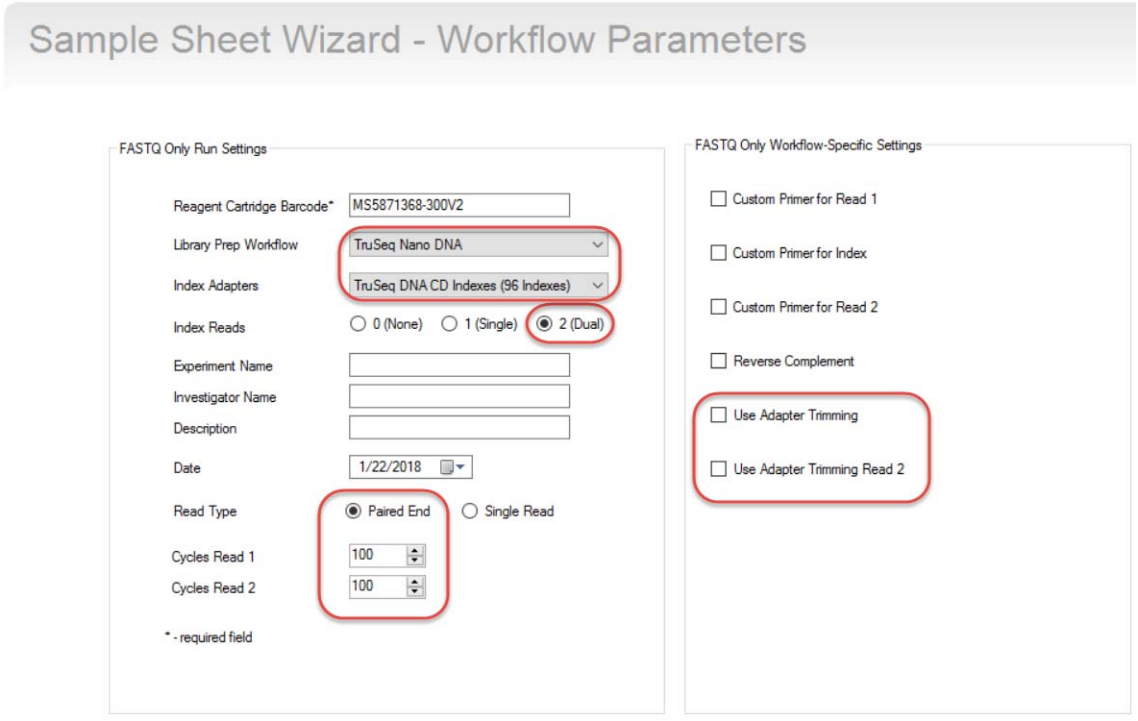
Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below. Once a Sample Sheet has been generated, index sequences need to be manually changed to the dual indexes used for each sample. See Table 49 on page 93 and Table 50 on page 94 for nucleotide sequences of the dual indexes.

Setting 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*.

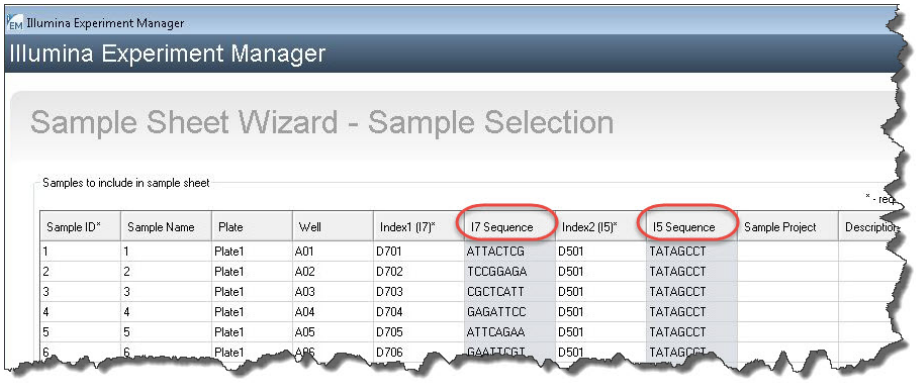
2 On the **Workflow Parameters** screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA**. In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. If your pipeline uses SureCall for adaptor trimming, then 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 i5 sequence from the SureSelect XT Low Input Dual Index P5 Indexed Adaptor 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.

Editing the Sample Sheet to include SureSelect XT Low Input dual indexes

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 below).

- 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 P7 index sequence. See Table 49 on page 93 for nucleotide sequences of the SureSelect XT Low Input indexes.
- In column 7 under **I5_Index_ID**, enter the name of the SureSelect XT Low Input Dual Index P5 Indexed Adaptor assigned to the sample. In column 8 under **index2**, enter the corresponding P5 index sequence. See Table 50 on page 94 for nucleotide sequences of the index segment of the SureSelect XT Low Input Dual Index P5 Indexed Adaptors.

[Header]									
IEMFileVe	5								
Experimer	XT_Low_Input								
Date	########								
Workflow	GenerateFASTQ								
Applicatic	FASTQ Only								
Instrumer	MiSeq								
Assay	TruSeq Nano DNA								
Index Ada	TruSeq DNA CD Indexes (96 Indexes)								
Description									
Chemistry	Amplicon								
[Reads]									
100									
100									
[Settings]									
ReverseCc	0								
[Data]									
Sample_II	Sample_N	Sample_P	Sample_W	Index_Pla	I7_Index_ID	index	I5_Index_ID	index2	Sample
Sample_1	Sample1	Plate1	A01	A01	A01	GTCTGTCA	A01	CAACGAGC	
Sample_2	Sample2	Plate1	B01	B01	B01	TGAAGAGA	B01	GTCGACAA	
Sample_3	Sample3	Plate1	C01	C01	C01	TTCACGCA	C01	AAGAGCCT	

Figure 15 Sample sheet for SureSelect XT Low Input dual indexed library sequencing

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

Sequence analysis resources

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

Agilent SureCall NGS data analysis software is designed to perform variant calling by analyzing the paired end reads. To download SureCall free-of-charge and for additional information, including SureCall software tutorials, visit www.agilent.com/surecall.

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 37.

Table 37 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 23	Qualification of DNA Integrity	Not required	Required
DNA input for Library Preparation page 23	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 24 and Table 9 on page 25)
DNA Shearing page 26	Mode of DNA Shearing	2 × 120 seconds	240 seconds (continuous)
Enzymatic Fragmentation of DNA page 29	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 41	Cycle number	8–11	11–14
Sequencing page 85	Output augmentation	Per project requirements	1× to 10× based on determined DNA integrity (see Table 38 and Table 39 on page 85)

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 38. 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 38 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 39. 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 39 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

Components supplied in the Agilent kits used in this protocol are detailed below.

Table 40 Contents of SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM p/n 5191-4056

Kit Component	Storage Condition	Format
SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM	–20°C	P5 Indexed Adaptors 1 through 96 (adaptor oligos containing 8-bp P5 index sequence), provided in green plate*

* See Table 48 on page 92 for a plate map and see Table 49 on page 93 or Table 50 on page 94 for index sequences.

CAUTION The SureSelect XT Low Input Dual Index P5 Indexed Adaptors 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 41 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 42 Contents of SureSelect XT Low Input Reagent Kit p/n G9703A

Component Kit Name	Storage Condition	Component Kit p/n
SureSelect XT Low Input Index Primers 1–96 for ILM (Pre PCR)	–20°C	5190-6444 (see Table 43)
SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0140 (see Table 44)
SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	Room Temperature	5190-9687 (see Table 45)
SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	–20°C	5190-9686 (see Table 46)

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

Table 43 SureSelect XT Low Input Index Primers for ILM Kits (Pre PCR) Content

Kit Component	Format
SureSelect XT Low Input Index Primers 1–96 for ILM	Reverse PCR primers containing 8-bp P7 index sequence 1 through 96, provided in yellow plate (Index Plate 1)*

* See Table 48 on page 92 for a plate map and see Table 49 on page 93 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 44 SureSelect XT HS and XT Low Input Library Preparation Kit (Pre PCR) Content

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

* The Adaptor Oligo Mix is not used in the dual indexed library preparation protocol described in this publication.

Table 45 SureSelect Target Enrichment Kit, ILM Hyb Module Box 1 (Post PCR) Content

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

Table 46 SureSelect XT HS and XT Low Input Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR) Content

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

Bundles of a SureSelect XT Low Input Reagent Kit with certain Target Enrichment Probes are available for purchase using the Agilent part numbers listed in Table 47. The SureSelect XT Low Input Reagent Kit included in these bundles is supplied with the same component kits listed in Table 42 on page 88.

Table 47 Supported SureSelect XT Low Input Reagent Kit + Probe Bundles

Included SureSelect (SSel) XT Low Input Probe Capture Library	Bundle part number
Custom 1–499 kb*	G9707A
Custom 0.5 –2.9 Mb*	G9707B
Custom 3–5.9 Mb*	G9707C
Custom 6–11.9 Mb*	G9707D
Custom 12–24 Mb*	G9707E
ClearSeq Comp Cancer	G9707G
Clinical Research Exome V2	G9707H
Clinical Research Exome V2 Plus	G9707J
Human All Exon V6	G9707K
Human All Exon V6 Plus	G9707L
Human All Exon V6+UTRs	G9707M
Human All Exon V7	G9707N
Human All Exon V7 Plus 1	G9707P
Human All Exon V7 Plus 2	G9707Q
Cancer All-In-One Lung	G9707R
Cancer All-In-One Solid Tumor	G9707S

* Kits that include Custom SureSelect Cancer All-In-One panels, designed using Agilent's SureDesign application, are ordered using these bundled custom design Agilent part numbers. Custom SureSelect Cancer All-In-One panels are designated using design IDs beginning with an 'A' character. (Refer to the probe vial label and the associated Certificate of Analysis to view the design ID.)

The plate map below shows the plate well position of each index for both P7 indexes in SureSelect XT Low Input Index Primers (yellow plate) and P5 indexes in SureSelect XT Low Input Dual Index P5 Indexed Adaptors (green plate). For each individual sample prepared using the dual indexing protocol, use the same P7 and P5 index number, originating from the same well position in the two plates. Use a different P7/P5 index pair for each sample to be multiplexed in the same sequencing reaction.

Table 48 Plate map for P7 indexes (yellow plate) and P5 indexes (green plate)

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

Nucleotide Sequences of SureSelect XT Low Input Dual Indexes

P7 Indexes (all platforms)

Table 49 P7 Indexes 1–96 (yellow 96-well plate)

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

P5 Indexes for NovaSeq (v 1.0 chemistry), MiSeq, or HiSeq2500 platform

Table 50 P5 Indexes 1–96 (green 96-well plate) for NovaSeq (v1.0 chemistry), MiSeq or HiSeq 2500

Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence
1	A01	CAACGAGC	25	A04	CCTGCGTG	49	A07	CGATACAG	73	A10	ACTCAGGC
2	B01	GTCGACAA	26	B04	TTCCAACA	50	B07	CCGTACTC	74	B10	GACCGCAT
3	C01	AAGAGCCT	27	C04	AGGCCTAG	51	C07	GTCGGTGT	75	C10	AGAGAGAA
4	D01	ACACCTTA	28	D04	AACGTGTC	52	D07	CTGACTAA	76	D10	TCAGATTG
5	E01	TGATCGCG	29	E04	GAGCCGCT	53	E07	ACTGGATG	77	E10	AAGATAGC
6	F01	TGGCTAGA	30	F04	ACATTACG	54	F07	TTGGCATG	78	F10	CGATTGGT
7	G01	GCCTCCGA	31	G04	TGCGACAT	55	G07	GACTCGTT	79	G10	GTTCCAAG
8	H01	CAGCGTTG	32	H04	TGTCCGGC	56	H07	CTCCGAAC	80	H10	CAATCTCG
9	A02	GTGTCTCA	33	A05	CTGTTCGC	57	A08	AATGGCAT	81	A11	CAAGTCAA
10	B02	ATAACATC	34	B05	TCACGCGA	58	B08	TAGCTGTA	82	B11	CAGTCGTG
11	C02	AACTTCCT	35	C05	GTTGTTCT	59	C08	GCTCACAC	83	C11	TTACAGTG
12	D02	GCGTTGGT	36	D05	ATTAACCG	60	D08	CTAATGTT	84	D11	ACCGGCCT
13	E02	CTAGCAAC	37	E05	CGTAGTAA	61	E08	CGTTACGT	85	E11	TCATTCCA
14	F02	TCTCGATC	38	F05	CACGCTGT	62	F08	GCGCATCA	86	F11	GCTAGGAT
15	G02	GTATGCGC	39	G05	TGGAACAG	63	G08	CCATCTAA	87	G11	ATGAATTG
16	H02	AGGTCGTT	40	H05	GTGTCGGC	64	H08	CGTCTCTT	88	H11	TTAGGCTC
17	A03	GTCAATAG	41	A06	AGAGTTCG	65	A09	ACCTTGTT	89	A12	TAACACCA
18	B03	CCTGTGAC	42	B06	TAGAACGC	66	B09	TATCGACG	90	B12	ACACTCTT
19	C03	GAGGAATA	43	C06	GAGATTAT	67	C09	TTGGCGAC	91	C12	CTGTATGA
20	D03	TGCTATCT	44	D06	TAATGAGA	68	D09	CGGAAGAT	92	D12	TTGGTCAA
21	E03	GATATCAC	45	E06	CTTGCCAA	69	E09	CAAGTATT	93	E12	CGTTGGCA
22	F03	CCTGAAGA	46	F06	CGCACAGA	70	F09	TGACGACT	94	F12	TCCACTTG
23	G03	TCTCTCAA	47	G06	GCGACTGT	71	G09	CGGCCATA	95	G12	AACGGTCA
24	H03	TTCCGTCT	48	H06	AGAATAAC	72	H09	TAAGTGGT	96	H12	CTGGACCA

P5 Indexes for NovaSeq (v 1.5 chemistry), NextSeq, or HiSeq 3000/4000 platform

Table 51 P5 Indexes 1–96 (green 96-well plate) for NovaSeq (v1.5 chemistry), NextSeq* or HiSeq 3000/4000

Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence
1	A01	GCTCGTTG	25	A04	CACGCAGG	49	A07	CTGTATCG	73	A10	GCCTGAGT
2	B01	TTGTCGAC	26	B04	TGTTGGAA	50	B07	GAGTACGG	74	B10	ATGCGGTC
3	C01	AGGCTCTT	27	C04	CTAGGCCT	51	C07	ACACCGAC	75	C10	TTCTCTCT
4	D01	TAAGGTGT	28	D04	GACACGTT	52	D07	TTAGTCAG	76	D10	CAATCTGA
5	E01	CGCGATCA	29	E04	AGCGGCTC	53	E07	CATCCAGT	77	E10	GCTATCTT
6	F01	TCTAGCCA	30	F04	CGTAATGT	54	F07	CATGCCAA	78	F10	ACCAATCG
7	G01	TCGGAGGC	31	G04	ATGTCGCA	55	G07	AACGAGTC	79	G10	CTTGGAAC
8	H01	CAACGCTG	32	H04	GCCGGACA	56	H07	GTTCGGAG	80	H10	CGAGATTG
9	A02	TGAGACAC	33	A05	GCGAACAG	57	A08	ATGCCATT	81	A11	TTGACTTG
10	B02	GATGTTAT	34	B05	TCGCGTGA	58	B08	TACAGCTA	82	B11	CACGACTG
11	C02	AGGAAGTT	35	C05	AGAACAAC	59	C08	GTGTGAGC	83	C11	CACTGTAA
12	D02	ACCAACGC	36	D05	CGGTTAAT	60	D08	AACATTAG	84	D11	AGGCCGGT
13	E02	GTTGCTAG	37	E05	TTACTACG	61	E08	ACGTAACG	85	E11	TGGAATGA
14	F02	GATCGAGA	38	F05	ACAGCGTG	62	F08	TGATGCGC	86	F11	ATCCTAGC
15	G02	GCGCATAC	39	G05	CTGTTCCA	63	G08	TTAGATGG	87	G11	CAATTCAT
16	H02	AACGACCT	40	H05	GCCGACAC	64	H08	AAGAGACG	88	H11	GAGCCTAA
17	A03	CTATTGAC	41	A06	CGAACTCT	65	A09	AACAAGGT	89	A12	TGGTGTTA
18	B03	GTCACAGG	42	B06	GCGTTCTA	66	B09	CGTCGATA	90	B12	AAGAGTGT
19	C03	TATTCCTC	43	C06	ATAATCTC	67	C09	GTCGCCAA	91	C12	TCATACAG
20	D03	AGATAGCA	44	D06	TCTCATTA	68	D09	ATCTTCCG	92	D12	TTGACCAA
21	E03	GTGATATC	45	E06	TTGGCAAG	69	E09	AATACTTG	93	E12	TGCCAACG
22	F03	TCTTCAGG	46	F06	TCTGTGCG	70	F09	AGTCGTCA	94	F12	CAAGTGGA
23	G03	TTGAGAGA	47	G06	ACAGTCGC	71	G09	TATGGCCG	95	G12	TGACCGTT
24	H03	AGACGGAA	48	H06	GTTATTCT	72	H09	ACCACTTA	96	H12	TGGTCCAG

* P5 Index sequences shown in this table are for the NextSeq platform without use of BaseSpace. If using BaseSpace, see the P5 Index sequences shown in Table 50.

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 30.
  - Add 3 µl of the master mix to each 17-µl DNA sample. Mix and spin as directed on page 30.
  - Run the thermal cycling program in Table 11 on page 29 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 43.

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 46 to page 49. 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 37. In particular, ensure that the Ligation master mix is mixed with the sample prior to adding the SureSelect XT Low Input Dual Index P5 Indexed Adaptor to the mixture. Do not add the Ligation master mix and the P5 Indexed Adaptor 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 43.

If yield of post-capture libraries is low

- ✔ PCR cycle number may require optimization. Repeat library preparation and target enrichment for the sample, increasing the post-capture PCR cycle number by 1 to 2 cycles.
- ✔ The RNA Capture Library used for hybridization may have been compromised. Verify the expiration date on the Capture Library vial or Certificate of Analysis. Adhere to the recommended storage and handling conditions. Ensure that the Capture Library Hybridization Mix is prepared immediately before use, as directed on page 55, and that solutions containing the Capture Library 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 65.

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 58)
  - Samples are maintained at 70°C during washes (see page 59)
  - Bead suspensions are mixed thoroughly during washes by pipetting up and down **and** vortexing (see page 59)
- ✔ Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a vortex and plate spinner or centrifuge in close proximity to thermal cycler to retain the 65°C sample temperature during mixing and transfer steps (step 8 to step 9 on page 56).

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 24 on page 53).

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 80 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 24 and page 25 Mechanically shear DNA using Covaris with shearing conditions on page 27 OR fragment DNA using SureSelect Enzymatic Fragmentation Kit with protocol on page 29 (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 P5 indexed adaptor	70 µl DNA sample + 25 µl Ligation master mix + 5 µl assigned P5 Indexed Adaptor (green plate) 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 and add P7 index	34.5 µl purified DNA + 13.5 µl PCR master mix + 2 µl assigned SureSelect XT Low Input Index Primer (yellow plate) Amplify in thermal cycler using program on page 41
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 using Agilent 2100 Bioanalyzer or 4200/4150 TapeStation instrument

Step	Summary of Conditions
	Hybridization/Capture
Program thermal cycler	Input thermal cycler program on page 53 and pause program
Prep DNA in hyb plate	Adjust 500–1000 ng purified prepared library to 12 µl volume with nuclease-free H_2O
Run pre-hybridization blocking protocol	12 µl library DNA + 5 µl SureSelect XT 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 Capture Library Hyb Mix	Prepare 25% RNAse Block dilution, then prepare appropriate mixture below: **Capture Libraries ≥3 Mb:** 2 µl 25% RNase Block + 5 µl Capture Library + 6 µl SureSelect Fast Hybridization Buffer **Capture Libraries <3 Mb:** 2 µl 25% RNase Block + 2 µl Capture Library + 3 µl nuclease-free H_2O + 6 µl SureSelect Fast Hybridization Buffer
Run the hybridization	With cycler paused and samples retained in cycler, add 13 µl Capture Library Hyb Mix to wells Resume the thermal cycler program, completing segments 4 (hybridization) and 5 (65°C hold)
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 63
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 nuclease-free H_2O
Quantify and qualify DNA	Analyze using Agilent 2100 Bioanalyzer or 4200/4150 TapeStation instrument

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In This Book

This guide contains information to run the SureSelect^{XT} Low Input target enrichment protocol with dual indexing.

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