SureSelect^{XT} Low Input Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library

For Illumina Multiplexed Sequencing Platforms

Protocol

Version C0, September 2018

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 target-enriched Illumina paired-end multiplexed sequencing libraries using SureSelect^{XT} Low Input Reagent Kits.

The SureSelect^{XT} Low Input Reagent Kits and protocol support molecular barcoding of indexed library samples prior to target enrichment and amplification.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Preparation

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

3 Hybridization and Capture

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

4 Post-Capture Sample Processing for Multiplexed Sequencing

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

5 Appendix: Using FFPE-derived DNA Samples

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

6 Reference

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

What's New in Version C0

- Support for use of Agilent's Enzymatic Fragmentation kits for DNA fragmentation, in place of mechanical DNA shearing (see footnote to Table 5 on page 17, and see page 19, page 20, page 23, and page 26)
- Addition of reagent mixture volumes for 24-reaction run sizes to reaction setup tables (see page 27, page 28, page 35, page 46, page 47, page 56)
- Updates to sequencing run setup instructions for the NextSeq platform (see page 68)
- Addition of *Note* to DNA shearing instructions (see page 25)
- Updates to Technical Support contact information (see page 2)
- Updates to Required Reagents (Table 2 on page 13) and Required Equipment (Table 5 on page 16) tables, including revised vendor/part number information for some materials purchased from Thermo Fisher Scientific or from general laboratory suppliers, and revised item order in tables
- Correction to description of vial formats in Table 11 on page 27 and Table 13 on page 28
- Correction of typographical error to well name D10 in Table 45 on page 88

What's New in Version B0

- Support for Human All Exon V7, Human All Exon V7 Plus 1, Human All Exon V7 Plus2, and Human All Exon V6+UTRs Capture Libraries (see Table 4 on page 15 and Table 41 on page 85)
- Revisions to sequencing support (see page 67 to page 74) including instructions for retrieval of I2 index files containing the molecular barcode (i5) index reads
- Vacuum concentrator removed from Required Materials table (see Table 5 on page 16)

- Updates to Table 10 on page 26 including increased duration of buffer thawing times and thawing of Adaptor Oligo Mix
- Updates to recommended duration of mixing for both Ligation Buffer and End Repair-A Tailing Buffer to 15 seconds (see page 27 and page 28)
- Update to component preparation instructions to include earlier transfer of AMPure XP beads to room temperature (see page 26)
- Addition of mixing instructions to PCR reagent preparation tables (see Table 15 on page 33 and Table 26 on page 54)
- Addition of optional stopping point after adaptor ligation step (see page 30)
- Updates to Technical Support contact information (see page 2)

Content

1 Before You Begin

Make sure you have the most current protocol. Go to genomics.agilent.com and search for G9703-90000.

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

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

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

Overview of the Workflow

The SureSelect^{XT} Low Input target enrichment workflow is summarized in Figure 1. The estimated time requirements for each step are summarized in Table 1.

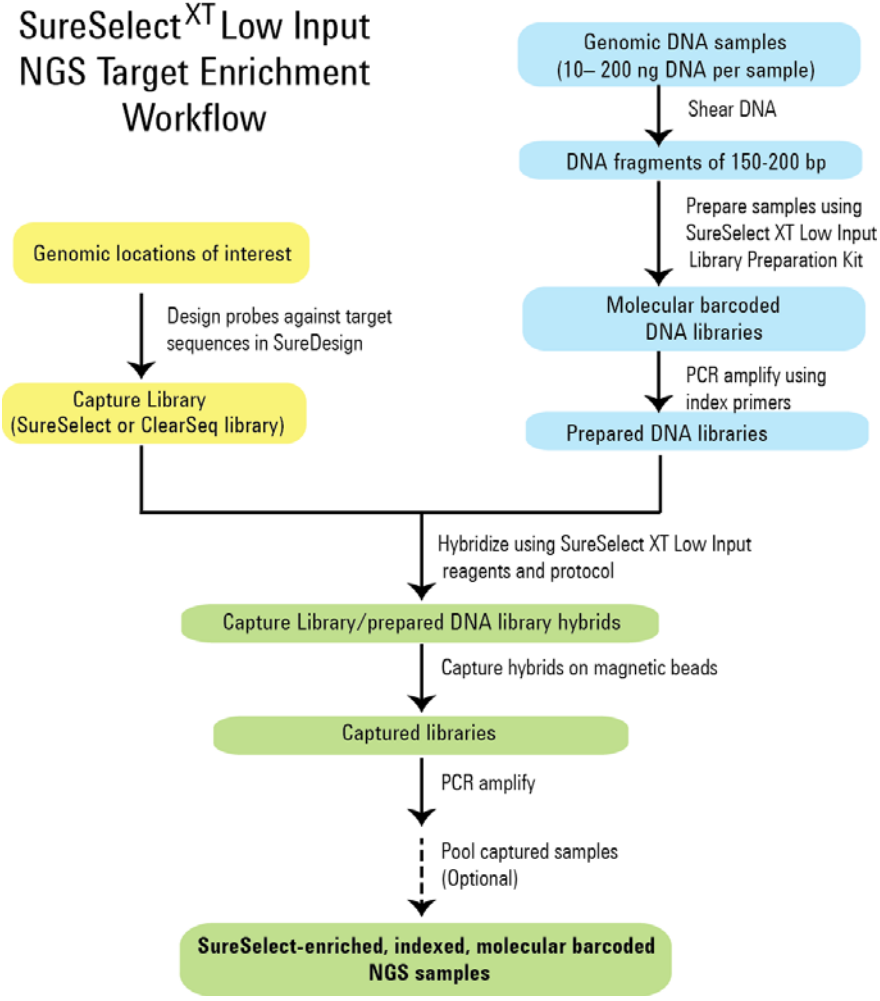


Figure 1 Overall target-enriched sequencing sample preparation workflow.

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

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

Procedural Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Use best-practices to prevent PCR product contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow.
  2. Maintain clean work areas. Clean pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution.
  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 domed caps. Cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler and from other procedural steps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during thermal cycler incubation steps.
- In general, follow Biosafety Level 1 (BL1) 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.

Required Reagents

Table 2 Required Reagents--All Sample Types

Description	Vendor and part number
SureSelect XT Low Input Reagent + Capture Library Kit for Illumina (ILM) platforms*	Select one kit from Table 4
96 reactions†, with Index Primers 1–96	p/n G9707A through G9707Q
96 reactions†, with Index Primers 97–192	p/n G9708A through G9708Q
Agencourt AMPure XP Kit	Beckman Coulter Genomics
5 ml	p/n A63880
60 ml	p/n A63881
450 ml	p/n A63882
Dynabeads MyOne Streptavidin T1	Thermo Fisher Scientific
2 ml	p/n 65601
10 ml	p/n 65602
100 ml	p/n 65603
1X Low TE Buffer (10 mM Tris-HCl, pH 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	Thermo Fisher Scientific
100 assays	p/n Q32850
500 assays	p/n Q32853
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

* Compatible with HiSeq, MiSeq and NextSeq 500 platforms.

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

Table 3 Required Reagents--FFPE DNA Samples Only

Description	Vendor and part number
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
QIAamp DNA FFPE Tissue Kit, 50 Samples	Qiagen p/n 56404
Deparaffinization Solution	Qiagen p/n 19093

Table 4 SureSelect^{XT} Low Input Reagent + Capture Library Kits

Included SureSelect (SSel) XT Low Input Capture Library	Included SureSelect^{XT} Low Input Reagent Kit	
	96 Reactions, with Index Primers 1–96	96 Reactions, with Index Primers 97–192
Custom 1–499 kb	G9707A	G9708A
Custom 0.5 –2.9 Mb	G9707B	G9708B
Custom 3–5.9 Mb	G9707C	G9708C
Custom 6–11.9 Mb	G9707D	G9708D
Custom 12–24 Mb	G9707E	G9708E
ClearSeq Comp Cancer	G9707G	G9708G
Clinical Research Exome V2	G9707H	G9708H
Clinical Research Exome V2 Plus	G9707J	G9708J
Human All Exon V6	G9707K	G9708K
Human All Exon V6 Plus	G9707L	G9708L
Human All Exon V6+UTRs	G9707M	G9708M
Human All Exon V7	G9707N	G9708N
Human All Exon V7 Plus 1	G9707P	G9708P
Human All Exon V7 Plus 2	G9707Q	G9708Q

Required Equipment

Table 5 Required Equipment

Description	Vendor and part number
SureCycler 8800 Thermal Cycler, or equivalent	Agilent p/n G8800A
96 well plate module for SureCycler 8800 Thermal Cycler	Agilent p/n G8810A
SureCycler 8800-compatible plasticware:	
96-well plates	Agilent p/n 410088
OR	
8-well strip tubes	Agilent p/n 410092
Tube cap strips, domed	Agilent p/n 410096
Qubit Fluorometer	Thermo Fisher Scientific p/n Q33226
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 5382 000.015 and Eppendorf SmartBlock 96 PCR, p/n 5306 000.006, 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

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
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†
Covaris Sample Preparation System‡	Covaris model E220
Covaris microTUBE sample holders‡	Covaris p/n 520045
Ice bucket	general laboratory supplier
Powder-free gloves	general laboratory supplier

* DNA samples may also be analyzed using the Agilent 2200 TapeStation, p/n G2964AA or G2965AA. ScreenTape devices and associated reagents listed in this table are compatible with both platforms.

† 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.

‡ This DNA shearing equipment is not required when DNA samples are enzymatically fragmented using Agilent's SureSelect XT HS Enzymatic Fragmentation Kit (p/n 5191-4079) or SureSelect XT HS and XT Low Input Enzymatic Fragmentation Kit (p/n 5191-4080).

Optional Reagents and Equipment

Table 6 Supplier Information for Optional Materials

Description	Vendor and part number
High-quality gDNA purification system, for example:	
QIAamp DNA Mini Kit 50 Samples 250 Samples	Qiagen p/n 51304 p/n 51306
Ethylene Glycol	American Bioanalytical p/n AB00455
Tween 20	Sigma-Aldrich p/n P9416-50ML
Optical Caps, 8× strip (flat)	Agilent p/n 401425*
Tube-strip capping tool	Agilent p/n 410099
PlateLoc Thermal Microplate Sealer with Small Hotplate	Agilent p/n G5402A
Peelable Aluminum Seal for PlateLoc Sealer	Agilent p/n 24210-001
MicroAmp Clear Adhesive Film	Thermo Fisher Scientific p/n L12-20

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

2
Sample 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 indexed and molecular-barcoded library is prepared. For an overview of the SureSelect^{XT} Low Input target enrichment workflow, see Figure 1 on page 10.

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 5, "Appendix: Using FFPE-derived DNA Samples" on page 77.

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.

This protocol includes DNA fragmentation by mechanical shearing. As an alternative, Agilent offers reagents and protocols for enzymatic DNA fragmentation prior to SureSelect^{XT} Low Input library preparation (see Agilent publication G9702-90050 for details). If you have enzymatically-fragmented DNA ready for use, proceed directly to "Step 3. Repair and dA-Tail the DNA ends" on page 26.

Step 1. Prepare and analyze quality of genomic DNA samples

NOTE Instructions in this section are used to prepare gDNA samples for mechanical shearing. To instead prepare input gDNA for enzymatic fragmentation, follow the sample preparation instructions provided in the SureSelect XT HS and XT Low Input Enzymatic Fragmentation Kit Protocol (publication G9702-90050).

Preparation of high-quality gDNA from fresh biological samples

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

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

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

3 Prepare each DNA sample for the library preparation protocol by diluting 10 ng to 200 ng gDNA with 1X Low TE Buffer to a final volume of 50 µl. Vortex well to mix, then spin briefly to collect the liquid. Keep the samples on ice.

Additional qualification of DNA samples is not required for DNA derived from fresh biological samples. Proceed to "Step 2. Shear the DNA" on page 23.

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 7.

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

b Remove a 1 µl aliquot of the FFPE gDNA sample for analysis using the Agilent NGS FFPE QC Kit to determine the ΔΔCq DNA integrity score. See the kit user manual at www.genomics.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 7 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 4200 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.genomics.agilent.com for more information.

c Using the DIN score reported for each sample in the Genomic DNA ScreenTape assay, consult Table 8 to determine the recommended amount of input DNA for the sample.

Table 8 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.

3 Prepare each FFPE DNA sample for the library preparation protocol by diluting the appropriate amount of gDNA with 1X Low TE Buffer to a final volume of 50 µl. See Table 7 or Table 8 above for FFPE DNA input guidelines based on the measured DNA quality in each sample.

Vortex each sample dilution to mix, then spin briefly to collect the liquid. Keep the samples on ice.

Step 2. Shear the DNA

NOTE The DNA shearing steps in this section may be replaced by enzymatic DNA fragmentation, using Agilent's SureSelect XT HS and XT Low Input Enzymatic Fragmentation Kits (see Protocol publication G9702-90050 for details).

In this step, the 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. Consult the manufacturer's recommendations for use of other Covaris instruments or sample holders to achieve the same target DNA fragment size.

1 Set up the Covaris E220 instrument.

- **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 for least 30 minutes before use, or according to the manufacturer's recommendations.
- **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.
- **e** *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.

Refer to the instrument user guide for more details.

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

Each high-quality DNA sample or FFPE DNA sample should contain 10–200 ng gDNA (adjusted as required for DNA integrity) in 50 µl of 1X Low TE Buffer.

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 septa 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 9.

Table 9 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 septa, 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.

Step 3. Repair and dA-Tail the DNA ends

The NGS library preparation protocol that begins here can be used for fragmented DNA samples produced by mechanical shearing (as detailed on page 20 to page 25) or produced by enzymatic fragmentation (as detailed in publication G9702-90050). Samples produced by either method should contain 10–200 ng of DNA fragments in a volume of 50 µl.

This step uses the components listed in Table 10. Thaw and mix each component as directed in Table 10 before use.

Remove the Agencort AMPure XP beads from cold storage and equilibrate to room temperature in preparation for use on page 31.

Table 10 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 28
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 27
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 28
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 27
Adaptor Oligo Mix (white cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	page 30

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.

   b Prepare the appropriate volume of Ligation master mix by combining the reagents in Table 11.

   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. Spin briefly to collect the liquid.

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

Table 11 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 SureCycler 8800 thermal cycler (with the heated lid ON) for the End Repair and dA-Tailing steps with the program in Table 12. Start the program, then immediately press the *Pause* button, allowing the heated lid to reach temperature while you set up the reactions.

Table 12 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 by pipetting up and down 15–20 times using a pipette set to at least 80% of the mixture volume.

4 Prepare the appropriate volume of End Repair/dA-Tailing master mix, by combining the reagents in Table 13.

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. Spin briefly to collect the liquid and keep on ice.

Table 13 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 of fragmented DNA. Mix by pipetting up and down 15–20 times using a pipette set to 60 µl.

6 Briefly spin the samples, then immediately place the plate or strip tube in the SureCycler 8800 thermal cycler. Press the *Play* button to resume the thermal cycling program in Table 12.

Step 4. Ligate the molecular-barcoded adaptor

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

2 Preprogram a SureCycler 8800 thermal cycler (with the heated lid ON) for the Ligation step with the program in Table 14. Start the program, then immediately press the *Pause* button, allowing the heated lid to reach temperature while you set up the reactions.

Table 14 Thermal cycler program for Ligation*

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

* When setting up the thermal cycling program, use a reaction volume setting of 100 µL.

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 27 and kept at room temperature. Mix by pipetting up and down at least 10 times using a pipette set to 85 µl, then briefly spin the samples.

4 Add 5 µl of Adaptor Oligo Mix (white capped tube) to each sample. Mix by pipetting up and down 15–20 times using a pipette set to 85 µl.

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

5 Briefly spin the samples, then immediately place the plate or strip tube in the SureCycler 8800 thermal cycler. Press the *Play* button to resume the thermal cycling program in Table 14.

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

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

Step 5. 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 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 6. Amplify the adaptor-ligated library

This step uses the components listed in Table 15. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 15 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 35
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 35
100 mM dNTP Mix (green cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	page 35
Forward Primer (brown cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	page 35
SureSelect XT Low Input Index Primers	SureSelect XT Low Input Index Primers for ILM (Pre PCR),* –20°C	Vortexing	page 35

* Indexing primers are provided in 96-well plates containing either indexes 1–96 (yellow *Index Plate 1*) or indexes 97–192 (red *Index Plate 2*).

1 Determine the appropriate index assignments for each sample. See Table 44 and Table 45 in the "Reference" chapter for sequences of the 8 bp index portion of the SureSelect XT Low Input Index Primers used to amplify the DNA libraries in this step.

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

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

2 Preprogram a SureCycler 8800 thermal cycler (with the heated lid ON) with the program in Table 16. Start the program, then immediately press the *Pause* button, allowing the heated lid to reach temperature while you set up the reactions.

Table 16 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 17)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

* When setting up the thermal cycling program, use a reaction volume setting of 50 µL.

Table 17 Pre-capture PCR cycle number recommendations

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

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

CAUTION To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

3 Prepare the appropriate volume of pre-capture PCR reaction mix, as described in Table 18, on ice. Mix well on a vortex mixer.

Table 18 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**

4 Add 13.5 µl of the PCR reaction mixture prepared in Table 18 to each purified DNA library sample (34.5 µl) in the PCR plate wells.

5 Add 2 µl of the appropriate SureSelect XT Low Input Index Primer to each reaction.

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

6 Before adding the samples to the thermal cycler, bring the temperature of the thermal block to 98°C by pressing the *Play* button to resume the thermal cycling program in Table 16. 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 7. 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 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 8. 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 36.

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

Use a Bioanalyzer DNA 1000 chip and reagent kit. For more information to do this step, see the *Agilent DNA 1000 Kit Guide* at www.genomics.agilent.com.

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 39. See Troubleshooting information on page 91 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.

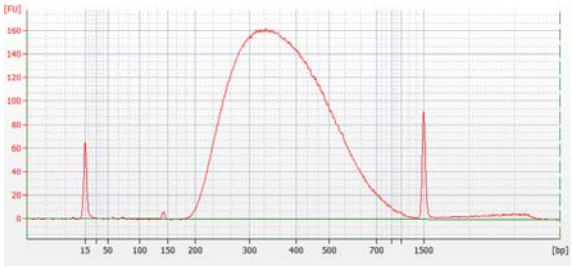


Figure 2 Pre-capture library prepared from a high-quality gDNA sample analyzed using a DNA 1000 Bioanalyzer assay.

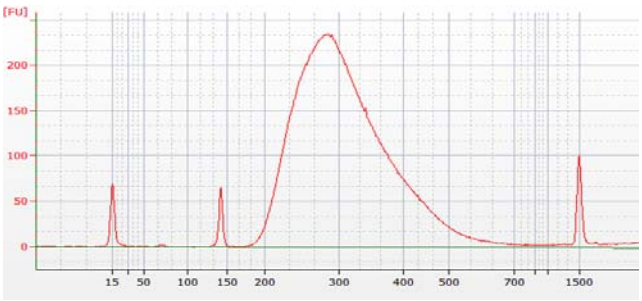


Figure 3 Pre-capture library prepared from a typical FFPE gDNA sample analyzed using a DNA 1000 Bioanalyzer assay.

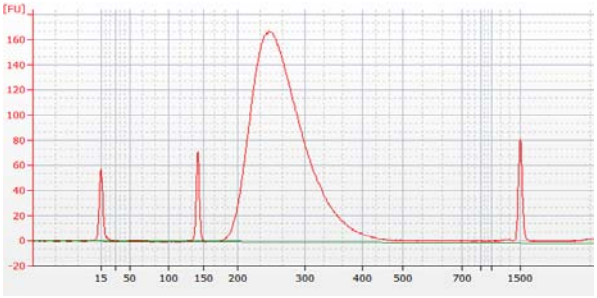


Figure 4 Pre-capture library prepared from a low-quality FFPE gDNA sample analyzed using a DNA 1000 Bioanalyzer assay.

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 4200 TapeStation or 2200 TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and associated reagent kit. For more information to do this step, see the appropriate TapeStation user manual at www.genomics.agilent.com.

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 Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

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 41 to page 42. See Troubleshooting information on page 91 for additional considerations.

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

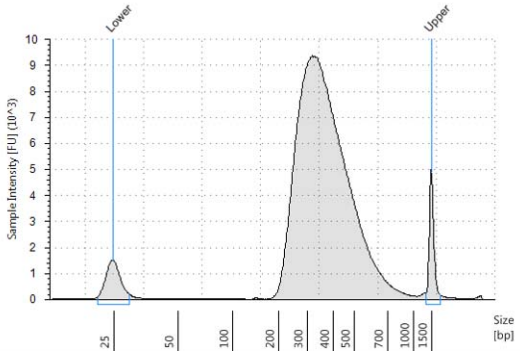


Figure 5 Pre-capture library prepared from a high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

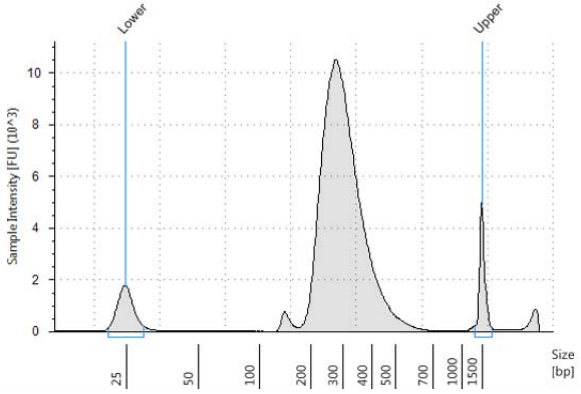


Figure 6 Pre-capture library prepared from a typical FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

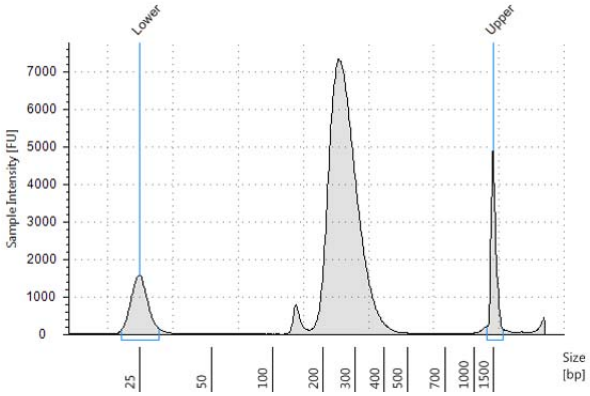


Figure 7 Pre-capture library prepared from a low-quality FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

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.

3
Hybridization and Capture

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

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

Step 1. Hybridize DNA samples to the Capture Library

In this step, the prepared gDNA libraries are hybridized to a target-specific 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 19. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 19 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 45
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 46
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 47
Capture Library	–80°C	Thaw on ice	page 47

1 Preprogram a SureCycler 8800 thermal cycler (with the heated lid ON) with the program in Table 20. Start the program, then immediately press the *Pause* button, allowing the heated lid to reach temperature while you set up the reactions.

Table 20 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).

† Reducing the hybridization temperature to 60°C (Segments 4 and 5) may improve coverage for AT-rich regions of some libraries.

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. 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.

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 press the *Play* button to resume the thermal cycling program set up on page 45 and shown in Table 21 below.

Important: Notice that the thermal cycler must be paused during Segment 3 (see Table 21) to allow additional reagents to be added to the Hybridization wells, as described in step 7 on page 48.

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 47. If needed, you can finish these preparation steps after pausing the thermal cycler in Segment 3.

Table 21 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 49 when the thermal cycler starts the 65°C Hold segment.

5 Prepare a 25% solution of SureSelect RNase Block (containing 1 part RNase Block:3 parts water), according to Table 22. Prepare the amount required for the number of hybridization reactions in the run, plus excess. Mix well and keep on ice.

Table 22 Preparation of RNase Block solution

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

NOTE

Prepare the mixture described in step 6, below, just before pausing the thermal cycler in Segment 3 as described on page 46. Keep the mixture at room temperature briefly until the mixture is added to the DNA samples in step 7 on page 48. Do not keep solutions containing the Capture Library at room temperature for extended periods.

6 Prepare the Capture Library Hybridization Mix appropriate for your capture library type according to Table 23 for Capture Libraries ≥3 Mb or Table 24 for Capture Libraries <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 23 Preparation of Capture Library Hybridization Mix for **Capture Libraries ≥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
Capture Library ≥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 24 Preparation of Capture Library Hybridization Mix for **Capture Libraries <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
Capture Library <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 21 (1 minute at 65°C), press the *Pause* button. 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 Press the *Play* button to resume the thermal cycling program to allow hybridization of the prepared DNA samples to the Capture Library.

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 25.

Begin the bead preparation steps described below approximately one hour after starting hybridization in step 9 on page 48.

Table 25 Reagents for Capture

Kit Component	Storage Location	Where Used
SureSelect Binding Buffer	SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), RT	page 49
SureSelect Wash Buffer 1	SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR),* RT	page 50
SureSelect Wash Buffer 2	SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR),* RT	page 50
Dynabeads MyOne Streptavidin T1	Follow storage recommendations provided by supplier (see Table 2 on page 13)	page 49

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 SureCycler 8800 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.
   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 the hybridization step is complete and the thermal cycler reaches the 65°C hold step (see Table 21 on page 46), 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 the supernatant.
7. Resuspend the beads in 200 µl of SureSelect Wash Buffer 1. Mix by pipetting up and down 15–20 times, until beads are fully resuspended.
8. Put the plate or strip tube in the magnetic separator. Wait for the solution to clear (approximately 1 minute), then remove and discard the supernatant.

CAUTION

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

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

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

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

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

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

Make sure the beads are in suspension before proceeding.

c Incubate the samples for 5 minutes at 70°C on the SureCycler 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 56.

NOTE

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

4 Post-Capture Sample Processing for Multiplexed Sequencing

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

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

Table 26 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 56
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 56
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 56
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 56

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 SureCycler 8800 thermal cycler (with the heated lid ON) with the program in Table 27. Start the program, then immediately press the *Pause* button, allowing the heated lid to reach temperature while you set up the reactions.

Table 27 Post-capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	9 to 14 See Table 28 for recommendations based on Capture Library size	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Table 28 Post-capture PCR cycle number recommendations

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

2 Prepare the appropriate volume of PCR reaction mix, as described in Table 29, on ice. Mix well on a vortex mixer.

Table 29 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 29 to each sample well containing 25 µl of bead-bound target-enriched DNA (prepared on page 51 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 SureCycler 8800 thermal cycler. Press the *Play* button to resume the thermal cycling program in Table 27.

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.

   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. See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information on doing this step.

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.

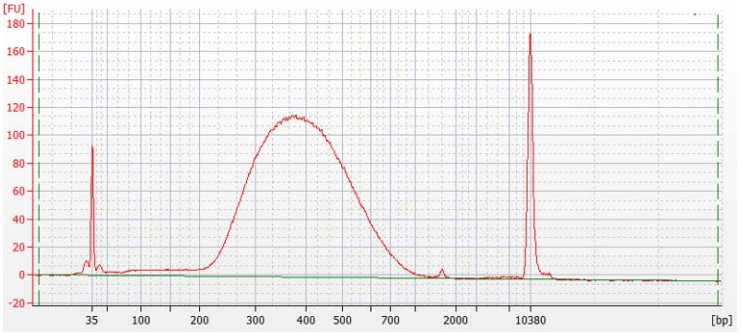


Figure 8 Post-capture library prepared from a high-quality gDNA sample analyzed using a Bioanalyzer system High Sensitivity DNA assay.

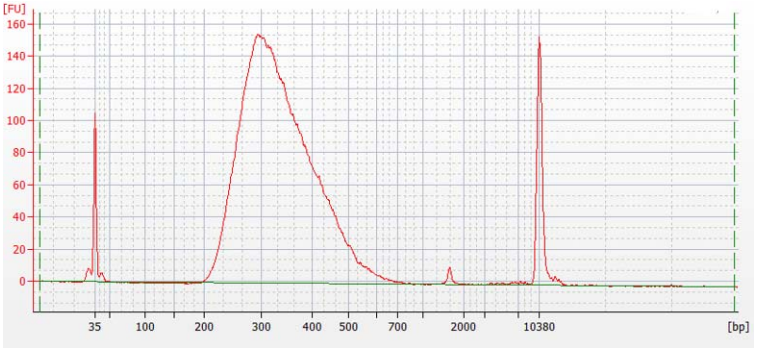


Figure 9 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a Bioanalyzer system High Sensitivity DNA assay.

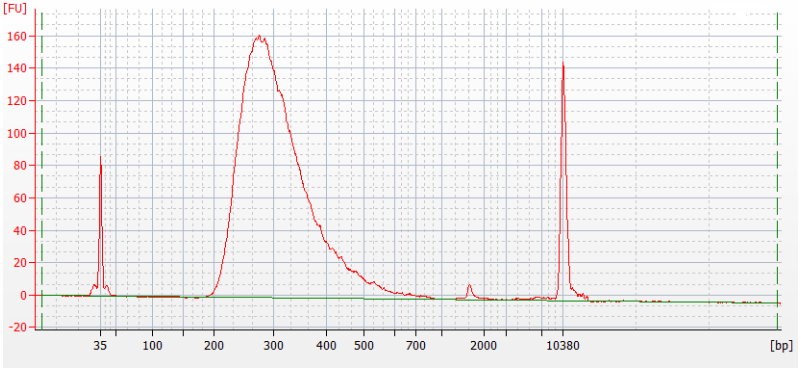


Figure 10 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a Bioanalyzer system High Sensitivity DNA assay.

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 4200 TapeStation or 2200 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 appropriate TapeStation user manual at www.genomics.agilent.com.

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

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

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 instrument user manual. 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.

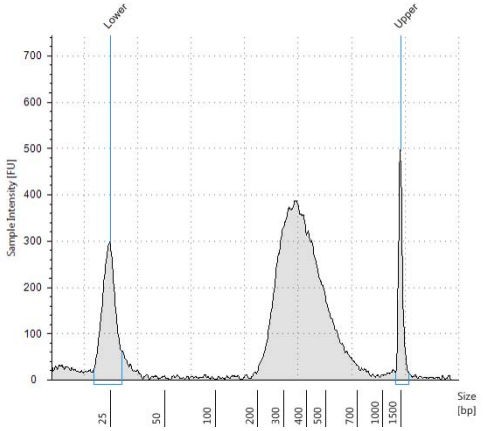


Figure 11 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

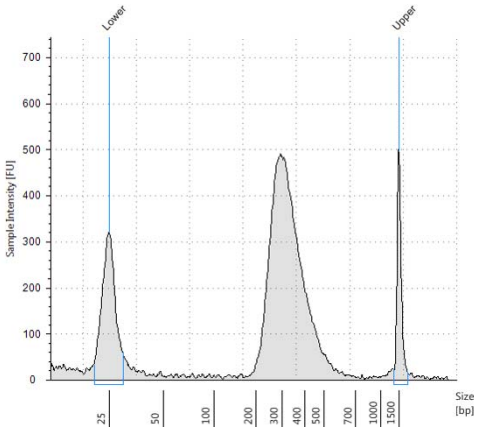


Figure 12 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

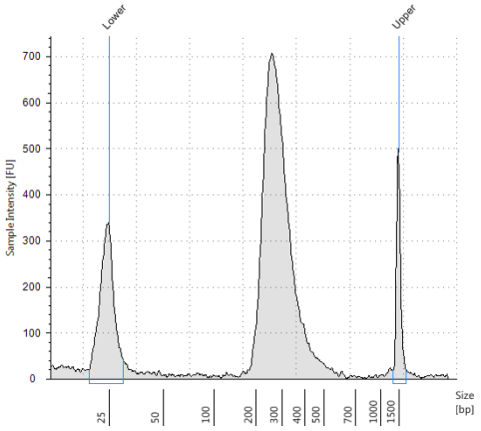


Figure 13 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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

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

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

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

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

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

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

is the number of indexes, and

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

Table 30 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µl at 10 nM DNA.

Table 30 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.

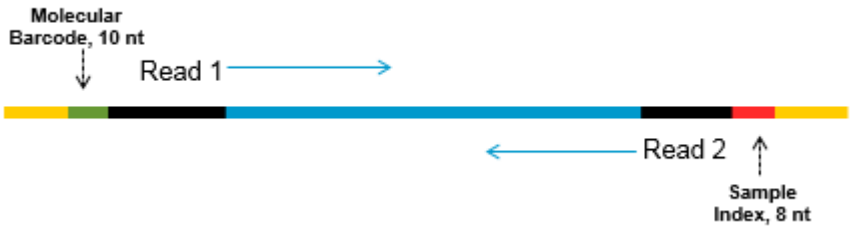


Figure 14 Content of SureSelect XT Low Input sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the sample index (red), the molecular barcode (green) and the library bridge PCR primers (yellow).

Libraries can be sequenced on the Illumina HiSeq, MiSeq, or NextSeq platform using the run type and chemistry combinations shown in Table 31.

CAUTION

Reduced molecular barcode 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. Please contact Agilent Technical Support for further information.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See Table 31 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 31 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.

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

Table 31 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	4×50 Cycle Kits*	v3	9–10 pM
HiSeq 2500	High Output†	2 × 100 bp	250 Cycle Kit	v4	12–14 pM
HiSeq 2000	All Runs	2 × 100 bp	4×50 Cycle Kits*	v3	6–9 pM
HiSeq 2000	All Runs	2 × 100 bp	250 Cycle Kit	v4	8–12 pM
MiSeq	All Runs	2 × 100 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	300 Cycle Kit	v2	1.5–1.8 pM
HiSeq 3000/4000	All Runs	2 × 100 bp	300 Cycle Kit	v1	180–190 pM

* A single 200-cycle kit does not include enough reagents to complete Reads 1 and 2 in addition to the 8-bp i7 and 10-bp i5 index reads in this format. If preferred, the additional reads may be supported by using one 200-cycle kit plus one 50-cycle kit.

† Reduced molecular barcode sequence quality and lowered Q scores have been observed in sequences obtained from HiSeq 2500 High Output (v4 chemistry) runs. Contact Agilent Technical Support for further information.

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.

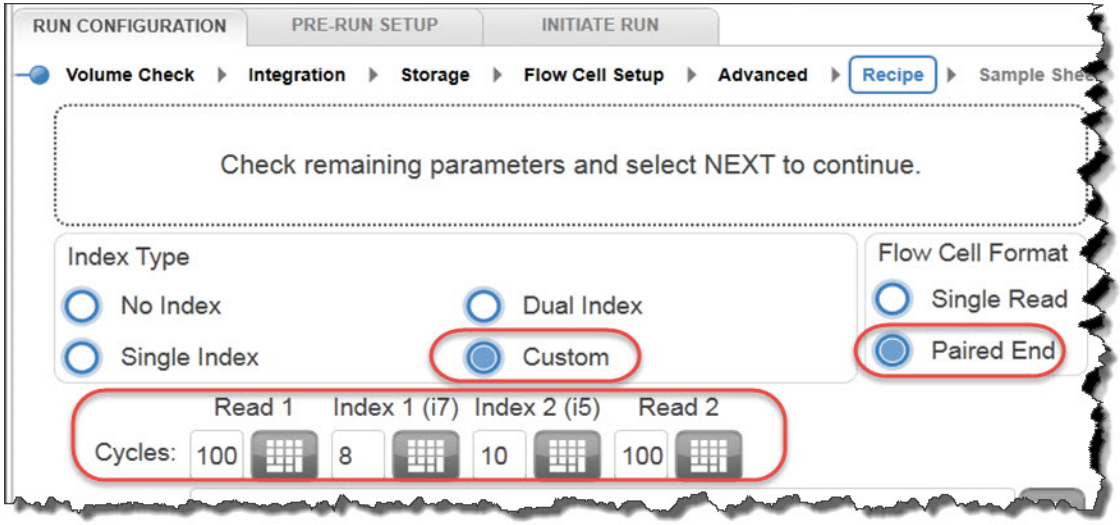
- The sample-level index (i7) requires an 8-bp index read. For complete i7 index sequence information, see Table 44 on page 87 and Table 45 on page 88.

CAUTION The 8-bp index sequences in SureSelect XT Low Input Index Primers 1-96 and 97-192 differ from the 8-bp index sequences in index primers A01 through H12 in Agilent's SureSelect XT system.

- The degenerate molecular barcode (i5) requires a 10-bp index read.
- For the HiSeq and NextSeq platforms, set up the run using the instrument's user interface, following the guidelines on page 68.
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed on page 71 to page 74 to generate a custom sample sheet.
- Retrieval of I2 index files containing the molecular barcode (i5) index reads requires offline conversion of .bcl to fastq files. For information on how to do this step, see page 68 for HiSeq and NextSeq runs and see page 74 for MiSeq runs.
- Before aligning reads to the reference genome, trim the reads from Illumina adaptor sequences. See page 74 for information on Agilent's SureCall data analysis software, which may be used for this task.

HiSeq and NextSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface. A sample run setup for the HiSeq platform using 100 + 100 bp paired-end sequencing is shown below.



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

BaseSpace currently does not support the sequencing of molecular barcodes as index reads. Set up NextSeq runs using the stand-alone mode.

Retrieve I2 FASTQ files containing molecular barcodes

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

Option 1: Use bcl2fastq software with base masking

To generate Index 2 fastq files containing the P5 molecular barcodes using the bcl2fastq software, follow Illumina's instructions for use of the software with the following modifications:

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

CAUTION

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

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

Option 2: Use Broad Institute Picard tools

To generate Index 2 fastq files containing the P5 molecular barcodes using the Broad Institute Picard tools, complete the following steps:

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

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

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

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

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

MiSeq platform sequencing run setup guidelines

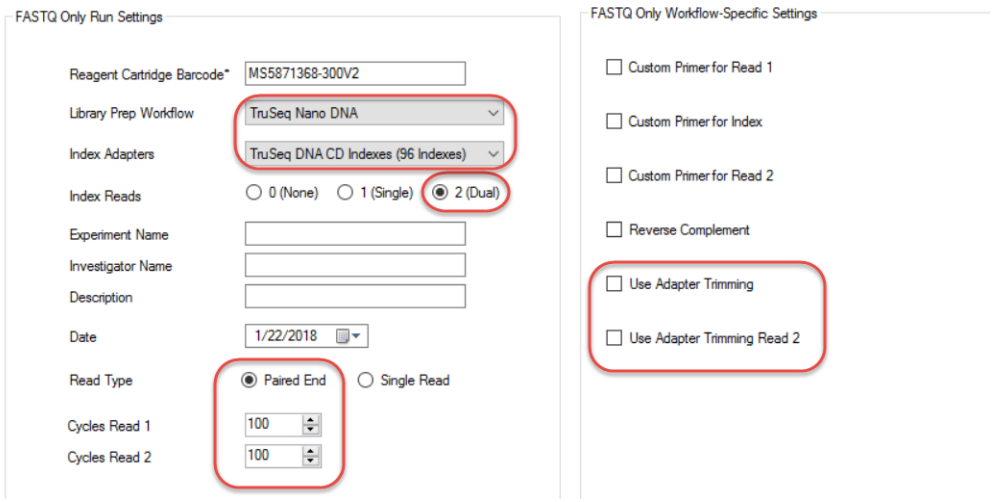
Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below. Once a Sample Sheet has been generated, index sequences need to be manually changed to the SureSelect Low Input indexes used for each sample. See Table 44 on page 87 and Table 45 on page 88 for nucleotide sequences of the SureSelect XT Low Input system indexes.

Set up a custom Sample Sheet:

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

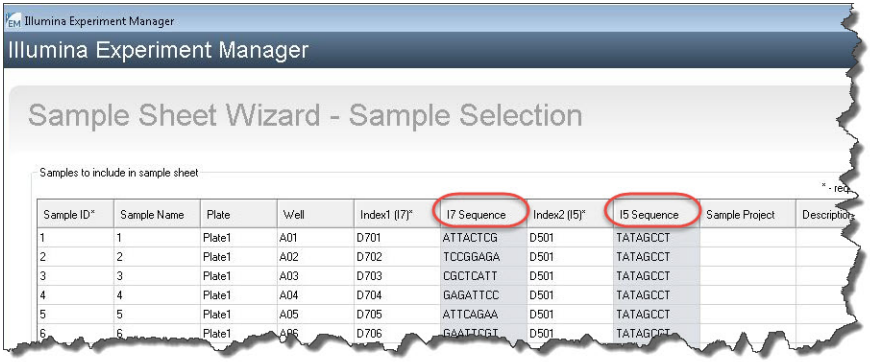
2 On the **Workflow Parameters** screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA**. In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. If your pipeline uses SureCall 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 degenerate molecular barcode at a later stage.



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

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

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

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

- In column 5 under **I7_Index_ID**, enter the name of the SureSelect XT Low Input index assigned to the sample. In column 6 under **index**, enter the corresponding SureSelect XT Low Input Index sequence. See Table 44 on page 87 and Table 45 on page 88 for nucleotide sequences of the SureSelect XT Low Input indexes.
- In column 7 under **I5_Index_ID**, enter *MBC* for all samples. In column 8 under **index2**, enter text *NNNNNNNNNN* for all samples to represent the degenerate 10-nucleotide molecular barcode tagging each fragment.

NOTE

Enter N_{10} text in the **index2** column only when sample sheets are processed using MiSeq Reporter software adjusted to retrieve I2 fastq files containing molecular barcodes, as detailed on page 74. Sample sheets processed offline using Illumina's bcl2fastq software must not contain N_{10} wildcard index sequences. See page 69 for more information.

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

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

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

Reconfigure the MiSeq Reporter Software to retrieve I2 FASTQ files

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

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

NOTE

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

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

The alternative methods for retrieval of I2 fastq files described on page 68 for HiSeq and NextSeq platform runs may also be applied to MiSeq platform runs.

Sequence analysis resources

Agilent SureCall NGS data analysis software is designed to perform adaptor trimming, alignment of reads, and variant calling of sequencing data generated from SureSelect^{XT} Low Input libraries. To download SureCall free-of-charge and for additional information, including SureCall software tutorials, visit www.agilent.com/genomics/surecall.

If using another pipeline for alignment and downstream analysis, Agilent provides the Agilent Genomics NextGen Toolkit (AGeNT), with certain of the Agilent SureCall capabilities in a flexible command-line interface for integration into your bioinformatics pipeline. AGeNT is a Java-based software module that has been designed to provide adaptor and low-quality bases trimming and duplicate read removal for high-sensitivity

(HS) and non-HS data. This tool is explicitly designed for users with established in-house bioinformatics experts with the capability to build, integrate, maintain, and troubleshoot internal analysis pipelines. Moreover, the module is designed specifically for users with sufficient computing infrastructure and IT support to troubleshoot all issues unrelated to the execution of the AGeNT algorithms. Because Agilent provides limited support of AGeNT, users with limited bioinformatics expertise should instead use Agilent SureCall software. Agilent does not guarantee the usability of third party tools (open- or closed-source) in upstream/downstream analysis of data in conjunction with AGeNT. For additional information on this tool, visit the AGeNT page at www.genomics.agilent.com.

5 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 32.

Table 32 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 21	Qualification of DNA Integrity	Not required	Required
DNA input for Library Preparation page 21	Input amount and means of quantification	10 ng to 200 ng, quantified by Qubit assay	Based on determined DNA integrity (see Table 7 on page 21and Table 8 on page 22)
DNA Shearing page 24	Mode of DNA Shearing	2 × 120 seconds	240 seconds (continuous)
Pre-capture PCR page 34	Cycle number	8–11	11–14
Sequencing page 79	Output augmentation	Per project requirements	1× to 10× based on determined DNA integrity (see Table 33 and Table 34 on page 79)

Methods for FFPE Sample Qualification

DNA integrity may be assessed using the Agilent NGS FFPE QC Kit or using the Agilent 4200 TapeStation system 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 4200 TapeStation system, combined with the Genomic DNA ScreenTape assay, provides a microfluidics-based 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 33. 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 33 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 34. 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 34 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

6 Reference

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

Kit Contents

SureSelect^{XT} Low Input Reagent + Capture Library Kits contain the following component kits:

Table 35 Contents of SureSelect^{XT} Low Input Reagent + Capture Library Kits p/n G9707A–Q (96 Reactions)

Component Kit Name	Storage Condition	Component Kit p/n
SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0140
SureSelect XT Low Input Index Primers 1–96 for ILM (Pre PCR)	–20°C	5190-6444
SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR)	Room Temperature	5190-9687
SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	–20°C	5190-9686
SSel XT HS and XT Low Input Capture Library	–80°C	see Table 41

Table 36 Contents of SureSelect^{XT} Low Input Reagent + Capture Library Kits p/n G9708A–Q (96 Reactions)

Component Kit Name	Storage Condition	Component Kit p/n
SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0140
SureSelect XT Low Input Index Primers 97–192 for ILM (Pre PCR)	–20°C	5190-6445
SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR)	Room Temperature	5190-9687
SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	–20°C	5190-9686
SSel XT HS and XT Low Input Capture Library	–80°C	see Table 41

The contents of each of the component kits listed in Table 35 and Table 36 are described in the tables below.

Table 37 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

Table 38 SureSelect XT Low Input Index Primers for ILM Kits (Pre PCR) Content

Kit Component	Index Primers 1-96 (p/n 5190-6444)	Index Primers 97-192 (p/n 5190-6445)
SureSelect XT Low Input Index Primers for ILM (reverse primers containing 8-bp index sequence)	Index Primers 1 through 96, provided in yellow plate (Index Plate 1)*	Index Primers 97 through 192, provided in red plate (Index Plate 2)†

* See Table 42 on page 86 for a plate map and see Table 44 on page 87 for index sequences.

† See Table 43 on page 86 for a plate map and see Table 45 on page 88 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 39 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 40 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

Table 41 Capture Library part numbers for kits G9707A–Q and G9708A–Q

Catalog No. Suffix	Capture Library	Part number
A	**SSel XT HS and XT Low Input Custom 1–499 kb**	5190-9927 (5190-9932 for reorder)
B	**SSel XT HS and XT Low Input Custom 0.5–2.9 Mb**	5190-9928 (5190-9933 for reorder)
C	**SSel XT HS and XT Low Input Custom 3–5.9 Mb**	5190-9929 (5190-9934 for reorder)
D	**SSel XT HS and XT Low Input Custom 6–11.9 Mb**	5190-9930 (5190-9935 for reorder)
E	**SSel XT HS and XT Low Input Custom 12–24 Mb**	5190-9931 (5190-9936 for reorder)
G	**SSel XT HS and XT Low Input ClearSeq Comp Cancer**	5190-9950
H	**SSel XT HS and XT Low Input Clinical Research Exome V2**	5190-9952
J	**SSel XT HS and XT Low Input Clinical Research Exome V2 Plus**	5190-9945 (5190-9946 for reorder)
K	**SSel XT HS and XT Low Input Human All Exon V6**	5190-9954
L	**SSel XT HS and XT Low Input Human All Exon V6 Plus**	5190-9947 (5190-9948 for reorder)
M	**SSel XT HS and XT Low Input Human All Exon V6+UTRs**	5190-9227
N	**SSel XT HS and XT Low Input Human All Exon V7**	5191-4029
P	**SSel XT HS and XT Low Input Human All Exon V7 Plus 1**	5191-4032 (5191-4050 for reorder)
Q	**SSel XT HS and XT Low Input Human All Exon V7 Plus 2**	5191-4035 (5191-4053 for reorder)

Table 42 Plate map for SureSelect XT Low Input Index Primers 1-96, provided in yellow plate (Index Plate 1)

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

Table 43 Plate map for SureSelect XT Low Input Index Primers 97-192, provided in red plate (Index Plate 2)

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

Nucleotide Sequences of SureSelect XT Low Input Indexes

Each index is 8 nt in length. See page 67 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 44 SureSelect XT Low Input Indexes 1–96, provided in yellow 96-well plate (Index Plate 1)

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

Table 45 SureSelect XT Low Input Indexes 97–192, provided in red 96-well plate (Index Plate 2)

Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence
97	A01	CCACACGA	121	A04	CTGTCAGT	145	A07	TCGAACGC	169	A10	GACCTCCT
98	B01	GACCACAC	122	B04	TCTAGTGT	146	B07	GCCTAAAT	170	B10	ACAAGGAC
99	C01	GTGCGACA	123	C04	GGATGATA	147	C07	CGTGATAA	171	C10	CCAAACCT
100	D01	GCTTCATG	124	D04	TACAGCGT	148	D07	TCCGCTGA	172	D10	CACCAGTT
101	E01	ACTAAGTC	125	E04	AGTACCGA	149	E07	GCTCATTG	173	E10	TGGACGAC
102	F01	CAGGAAAG	126	F04	GAGCCAAG	150	F07	AATCGATG	174	F10	GTTACAGC
103	G01	GATCCGCT	127	G04	AGCGACAT	151	G07	TTCCATCA	175	G10	GAACAATG
104	H01	GTATCAAC	128	H04	TTACCACC	152	H07	ATTCACAG	176	H10	CAATGACT
105	A02	TAGAGTCG	129	A05	AGACGCCA	153	A08	CGGAAAGA	177	A11	GCTCGAAC
106	B02	TCGACACT	130	B05	CATACTGG	154	B08	GTCAAGTG	178	B11	TCGGTAGC
107	C02	CTGACCTC	131	C05	CACGCATT	155	C08	CATCTTCA	179	C11	TACGAACT
108	D02	CATGGCTT	132	D05	TGGTCAAG	156	D08	GATAGGAT	180	D11	GCCGGATT
109	E02	GTACAGAT	133	E05	GACGGAAA	157	E08	CAAGTGGT	181	E11	TAGCTCGG
110	F02	TAGTGTTC	134	F05	AGTAGACT	158	F08	GCGTTACA	182	F11	TTGCCGGA
111	G02	ATCGAAAC	135	G05	TACAAAGG	159	G08	TATGCAAC	183	G11	GGTATGGT
112	H02	TCAAGTCA	136	H05	CGCAAGAT	160	H08	GAGACCGT	184	H11	TCACTAAG
113	A03	GGAACAAT	137	A06	TGTTGCAA	161	A09	TCGATGAA	185	A12	CCTCCCAT
114	B03	TAGCGAGT	138	B06	ATCAACGT	162	B09	TCAAAGAG	186	B12	GTTCTAGT
115	C03	TACCGAAG	139	C06	GACGACTG	163	C09	GTGGTATG	187	C12	GAGAAACC
116	D03	TAAGTCAC	140	D06	ACTGGACG	164	D09	CTGAGAAT	188	D12	CCTGTAAT
117	E03	ATAACGTG	141	E06	TGATAACG	165	E09	TCTATCCG	189	E12	CCTTACCA
118	F03	GGTAGCTC	142	F06	ACATAGCG	166	F09	GCAATGTT	190	F12	ATGATAGG
119	G03	GAAGTACC	143	G06	ACACAAGG	167	G09	CACATAGC	191	G12	TATGGTGG
120	H03	CAACGTAT	144	H06	GAACGCTC	168	H09	TCCTGACC	192	H12	TGAGGAAT

Troubleshooting Guide

If recovery of gDNA from samples is low

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

If yield of pre-capture libraries is low

- ✔ The library preparation protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance of the highly viscous buffer and enzyme solutions used in the protocol. Be sure to adhere to all instructions when setting up the reactions.
- ✔ Ensure that the ligation master mix (see page 27) 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 36.

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 39 to page 42. 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 30. In particular, ensure that the Ligation master mix is mixed with the sample prior to adding the Adaptor Oligo Mix to the mixture. Do not add the Ligation master mix and the Adaptor Oligo Mix to the sample in a single step.
- ✔ For whole-genome sequencing (not specifically supported by this protocol), samples with an adaptor-dimer peak must be subjected to an additional round of SPRI-purification. To complete, first dilute the sample to 50 µl with nuclease free water, then follow the SPRI purification procedure on page 36.

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 47, 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 57.

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 50)
- Samples are maintained at 70°C during washes (see page 51)
- Bead suspensions are mixed thoroughly during washes by pipetting up and down **and** vortexing (see page 51)

✔ 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 48).

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 20 on page 45).

Quick Reference Protocol

An abbreviated summary of the protocol steps is provided below for experienced users. Use the complete protocol on page 20 to page 74 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 and qualify DNA samples	Prepare 10–200 ng gDNA in 50 µl Low TE For FFPE DNA, qualify integrity and adjust input amount as directed on page 21 and page 22
Shear DNA	Use shearing conditions on page 24, with two rounds of duration 120 seconds for high-quality DNA and single round of duration of 240 seconds for FFPE DNA
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	50µl sheared DNA sample + 20 µl End Repair/dA-Tailing master mix Incubate in thermal cycler: 15 min @ 20°C, 15 min @ 72°C, Hold @ 4°C
Ligate adaptor	70 µl DNA sample + 25 µl Ligation master mix +5 µl Adaptor Oligo Mix Incubate in thermal cycler: 30 min @ 20°C, Hold @ 4°C
Purify DNA	100 µl DNA sample + 80 µl AMPure XP bead suspension Elute DNA in 35 µl nuclease-free H_2O
Prepare PCR master mix	Per reaction: 10 µl 5× Herculase II Reaction Buffer + 0.5 µl 100 mM dNTP Mix + 2 µl Forward Primer + 1 µl Herculase II Fusion DNA Polymerase Keep on ice
Amplify the purified DNA	34.5 µl purified DNA + 13.5 µl PCR master mix + 2 µl assigned SureSelect XT Low Input Index Primer Amplify in thermal cycler using program on page 34
Purify amplified DNA	50 µl amplified DNA + 50 µl AMPure XP bead suspension Elute DNA in 15 µl nuclease-free H_2O
Quantify and qualify DNA	Analyze 1 µl using Agilent 2100 Bioanalyzer or 4200 TapeStation instrument

Step	Summary of Conditions
	Hybridization/Capture
Program thermal cycler	Input thermal cycler program on page 45 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 55
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 1 µl using Agilent 2100 Bioanalyzer or 4200 TapeStation instrument

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In This Book

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

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Agilent Technologies