

SureSelect XT HS2 DNA System

DNA Library Preparation and Target Enrichment for the Illumina Platform

Protocol

Version D0, April 2021

SureSelect platform manufactured with Agilent SurePrint Technology

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In this Guide...

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

1 Before You Begin

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

2 Preparation and Fragmentation of Input DNA

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

3 Library Preparation

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

4 Hybridization and Capture

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

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

6 Appendix: Using FFPE-derived DNA Samples

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

7 Reference

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

What's New in Version D0

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

What's New in Version C1

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

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

What's New in Version C0

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

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

NOTE

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

NOTE

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



Overview of the Workflow

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

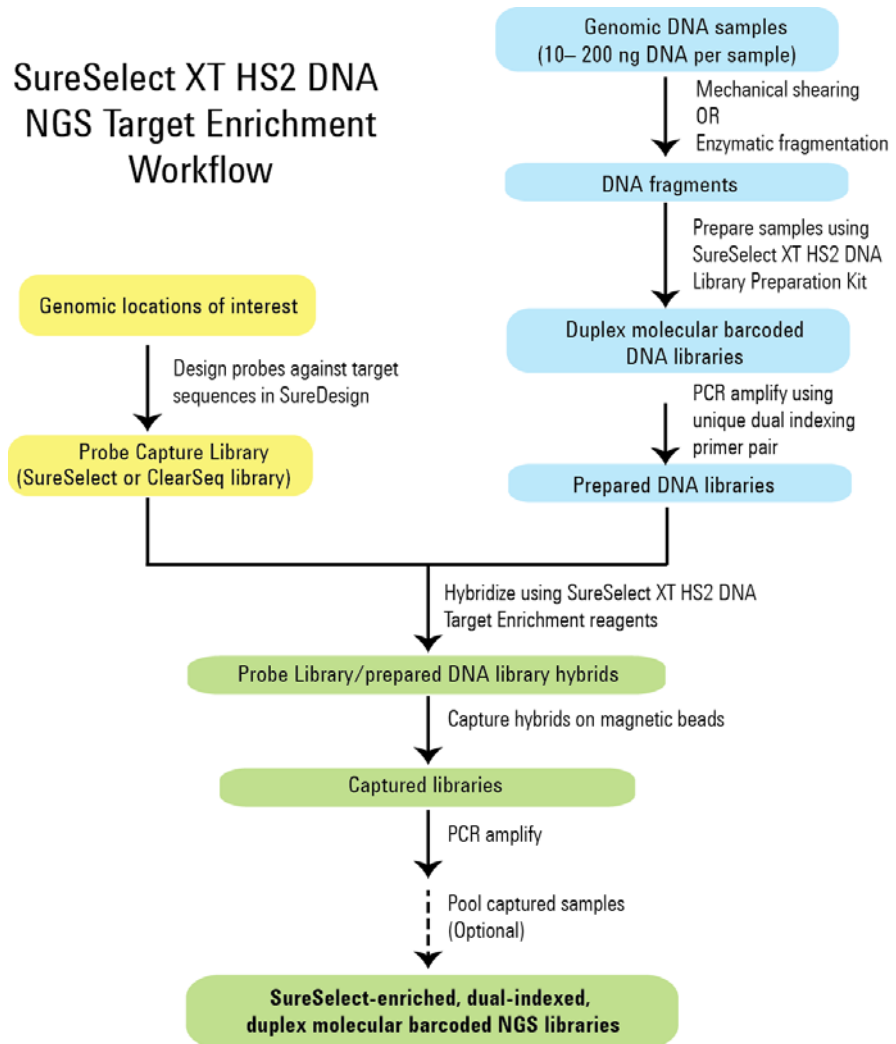


Figure 1 Overall target-enriched sequencing sample preparation workflow.

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

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

Procedural Notes

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

Safety Notes

CAUTION

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

Materials Required

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

- SureSelect XT HS2 DNA Reagent Kit format preference, where some options include ancillary reagent modules
- DNA sample type: high-quality gDNA derived from fresh/fresh-frozen samples vs. FFPE-derived gDNA samples
- DNA fragmentation method used in workflow: mechanical (Covaris-mediated) shearing vs. enzymatic fragmentation

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

Table 2 SureSelect XT HS2 DNA Reagent Kit Varieties

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

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

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

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

1 Before You Begin

Materials Required

Table 3 Compatible Probes

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

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

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

| Description | Vendor and Part Number | Notes |
|---|--|--|
| 1X Low TE Buffer | Thermo Fisher Scientific p/n 12090-015, or equivalent | 10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA |
| 100% Ethanol (Ethyl Alcohol, 200 proof) | Millipore p/n EX0276 | — |
| Qubit BR dsDNA Assay Kit 100 assays 500 assays | Thermo Fisher Scientific p/n Q32850 p/n Q32853 | — |
| Nuclease-free Water | Thermo Fisher Scientific p/n AM9930 | Water should not be DEPC-treated |
| AMPure [®] XP Kit 5 ml 60 ml 450 ml | Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882 | Separate purchase not required for use with SureSelect XT HS2 DNA Reagent Kits that include SureSelect DNA AMPure [®] XP Beads and SureSelect Streptavidin Beads (Agilent p/n G9982A, G9984A, G9984B, G9984C, or G9984D) |
| Dynabeads MyOne Streptavidin T1 2 ml 10 ml 50 ml | Thermo Fisher Scientific p/n 65601 p/n 65602 p/n 65604D | |

1 Before You Begin

Materials Required

CAUTION

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

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

| Description | Vendor and Part Number |
|--|--|
| Thermal Cycler with 96-well, 0.2 ml block | Various suppliers |
| Plasticware compatible with the selected thermal cycler: 96-well plates or 8-well strip tubes Tube cap strips, domed | Consult the thermal cycler manufacturer's recommendations |
| Nucleic acid analysis system (instrument and consumables) | Select one system from Table 6 on page 17 |
| Qubit Fluorometer | Thermo Fisher Scientific p/n Q33238 |
| Qubit Assay Tubes | Thermo Fisher Scientific p/n Q32856 |
| DNA LoBind Tubes, 1.5-ml PCR clean, 250 pieces | Eppendorf p/n 022431021 or equivalent |
| Microcentrifuge | Eppendorf microcentrifuge, model 5417C or equivalent |
| Plate or strip tube centrifuge | Labnet International MPS1000 Mini Plate Spinner, p/n C1000 (requires adapter, p/n C1000-ADAPT, for use with strip tubes) or equivalent |
| 96-well plate mixer | Eppendorf ThermoMixer C, p/n 5382000023 and Eppendorf SmartBlock 96 PCR, p/n 5306000006, or equivalent |
| Magnetic separator | Thermo Fisher Scientific p/n 12331D or equivalent* |
| Multichannel pipette | Rainin Pipet-Lite Multi Pipette or equivalent |
| Single channel pipettes (10-, 20-, 200-, and 1000- μ l capacity) | Rainin Pipet-Lite Pipettes or equivalent |
| Sterile, nuclease-free aerosol barrier pipette tips | general laboratory supplier |
| Vortex mixer | general laboratory supplier |
| Ice bucket | general laboratory supplier |
| Powder-free gloves | general laboratory supplier |

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

Table 6 Nucleic Acid Analysis Platform Options--Select One

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

1 Before You Begin

Materials Required

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

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

Optional Materials

Table 8 Supplier Information for Optional Materials

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

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

1 Before You Begin
Optional Materials



2 Preparation and Fragmentation of Input DNA

- Step 1. Prepare and analyze quality of genomic DNA samples 22
 - Preparation of high-quality gDNA from fresh biological samples 22
 - Preparation and qualification of gDNA from FFPE samples 22
- Step 2. Fragment the DNA 25
 - Method 1: Mechanical DNA Shearing using Covaris 25
 - Method 2: Enzymatic DNA Fragmentation 28

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

The library preparation protocol is compatible with both high-quality gDNA prepared from fresh or fresh-frozen samples and lower-quality DNA prepared from FFPE samples. Modifications required for FFPE samples are included throughout the protocol steps. For a summary of modifications for FFPE samples see [Chapter 6](#), “Appendix: Using FFPE-derived DNA Samples” on [page 79](#).

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



2 Preparation and Fragmentation of Input DNA

Step 1. Prepare and analyze quality of genomic DNA samples

Step 1. Prepare and analyze quality of genomic DNA samples

Preparation of high-quality gDNA from fresh biological samples

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

NOTE

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

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

Additional qualification of DNA samples is not required for DNA derived from fresh biological samples. Proceed to [“Step 2. Fragment the DNA”](#) on page 25.

Preparation and qualification of gDNA from FFPE samples

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

NOTE

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

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

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

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

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include a $\Delta\Delta Cq$ 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 $\Delta\Delta Cq$ scores for individual samples are summarized in Table 9.

- a Use the Qubit BR dsDNA Assay Kit to determine the concentration of each gDNA sample. Follow the manufacturer's instructions for the instrument and assay kit.
- b Remove a 1 μ l aliquot of the FFPE gDNA sample for analysis using the Agilent NGS FFPE QC Kit to determine the $\Delta\Delta Cq$ DNA integrity score. See the kit user manual at www.agilent.com for more information.
- c For all samples with $\Delta\Delta Cq$ 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 $\Delta\Delta Cq$ DNA integrity score > 1 , use the qPCR-based concentration of amplifiable gDNA, reported by the Agilent NGS FFPE QC Kit results, to determine amounts of input DNA for the protocol.

Table 9 SureSelect XT HS2 DNA input modifications based on $\Delta\Delta Cq$ DNA integrity score

| Protocol Parameter | non-FFPE Samples | FFPE Samples | |
|-----------------------------------|---|---|--|
| | | $\Delta\Delta Cq \leq 1^*$ | $\Delta\Delta Cq > 1$ |
| 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 $\Delta\Delta Cq$ 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.

2 Preparation and Fragmentation of Input DNA

Preparation and qualification of gDNA from FFPE samples

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

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

- a Use the Qubit BR dsDNA Assay Kit to determine the concentration of each gDNA sample. Follow the manufacturer's instructions for the instrument and assay kit.
- b Remove a 1 μ l aliquot of the FFPE gDNA sample and analyze using the Genomic DNA ScreenTape assay. See the user manual at www.agilent.com for more information.
- c Using the DIN score reported for each sample in the Genomic DNA ScreenTape assay, consult [Table 10](#) to determine the recommended amount of input DNA for the sample.

Table 10 SureSelect XT HS2 DNA input modifications based on DNA Integrity Number (DIN) score

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

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

Step 2. Fragment the DNA

Method 1: Mechanical DNA Shearing using Covaris

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

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

Table 11 Covaris shearing duration based on NGS read length

| NGS read length requirement | Target fragment size | Shearing duration for high-quality DNA samples | Shearing duration for FFPE DNA samples * |
|-----------------------------|----------------------|--|--|
| 2 \times 100 reads | 150 to 200 bp | 2 \times 120 seconds | 240 seconds |
| 2 \times 150 reads | 180 to 250 bp | 2 \times 60 seconds | 240 seconds |

* For FFPE DNA samples, initial DNA fragment size may impact the post-shear fragment size distribution, resulting in fragment sizes shorter than the target ranges listed in this table. All FFPE samples should be sheared for 240 seconds to generate fragment ends suitable for library construction. Libraries prepared from FFPE samples should be analyzed using an NGS read length suitable for the final library fragment size distribution.

NOTE

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

- 1 Set up the Covaris E220 instrument. Refer to the instrument user guide.
 - a Check that the water in the Covaris tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations.
 - b Check that the water covers the visible glass part of the tube.
 - c On the instrument control panel, push the Degas button. Degas the instrument according to the manufacturer's recommendations, typically 30–60 minutes.
 - d Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C. Consult the manufacturer's recommendations for addition of coolant fluids to prevent freezing.

2 Preparation and Fragmentation of Input DNA

Method 1: Mechanical DNA Shearing using Covaris

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

NOTE

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

- 3 Complete the DNA shearing steps below for each of the gDNA samples.
 - a Transfer the 50-µl DNA sample into a Covaris microTUBE, using a tapered pipette tip to slowly transfer the sample through the pre-split septum of the cap.
 - b Spin the microTUBE for 30 seconds to collect the liquid and to remove any bubbles from the bottom of the tube.
 - c Secure the microTUBE in the tube holder and shear the DNA with the settings in [Table 12](#).

Table 12 Shear settings for Covaris E-series instrument (SonoLab software v7 or later)

| Setting | High-quality DNA for 2 × 100 read NGS | High-quality DNA for 2 × 150 read NGS | FFPE DNA (2 × 100 or 2 × 150 read NGS) |
|---------------------------|--|--|---|
| Duty Factor | 10% | 10% | 10% |
| Peak Incident Power (PIP) | 175 | 175 | 175 |
| Cycles per Burst | 200 | 200 | 200 |
| Treatment Time | 2 × 120 seconds | 2 × 60 seconds | 240 seconds |
| Bath Temperature | 2° to 8° C | 2° to 8° C | 2° to 8° C |

- d Use the steps below for two-round shearing of **high-quality DNA samples only**:
 - Shear for 120 or 60 seconds (see [Table 12](#))
 - Spin the microTUBE for 10 seconds
 - Vortex the microTUBE at high speed for 5 seconds
 - Spin the microTUBE for 10 seconds
 - Shear for additional 120 or 60 seconds
 - Spin the microTUBE for 10 seconds
 - Vortex the microTUBE at high speed for 5 seconds
 - Spin the microTUBE for 10 seconds

- e** After completing the shearing step(s), put the Covaris microTUBE back into the loading and unloading station.
- f** While keeping the snap-cap on, insert a pipette tip through the pre-split septum, then slowly remove the sheared DNA.
- g** Transfer the sheared DNA sample (approximately 50 μ l) to a 96-well plate or strip tube sample well. Keep the samples on ice.
- h** After transferring the DNA sample, spin the microTUBE briefly to collect any residual sample volume. Transfer any additional collected liquid to the sample well used in [step g](#).

NOTE

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

The 50- μ l sheared DNA samples are now ready for NGS sequencing library preparation, beginning with end repair/dA-tailing. Proceed to “[Library Preparation](#)” on page 31.

NOTE

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

Method 2: Enzymatic DNA Fragmentation

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

- 1 In wells of a thermal cycler-compatible strip tube or PCR plate, dilute 10 ng to 200 ng of each gDNA sample with nuclease-free water to a final volume of 7 μ l.
- 2 Thaw the vial of 5X SureSelect Fragmentation Buffer, vortex, then place on ice.
- 3 Preprogram a thermal cycler with the program in [Table 13](#). Immediately pause the program, and keep paused until samples are loaded in [step 7](#).

Table 13 Thermal cycler program for enzymatic fragmentation *

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

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

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

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

| NGS read length requirement | Target fragment size | Duration of 37°C incubation step (Table 13) | |
|-----------------------------|----------------------|---|--------------------|
| | | High-quality DNA samples | FFPE DNA samples * |
| 2 × 100 reads | 150 to 200 bp | 15 minutes | 15 minutes |
| 2 × 150 reads | 180 to 250 bp | 10 minutes | 15 minutes |

* For FFPE DNA samples, initial DNA fragment size may impact the post-fragmentation size distribution, resulting in fragment sizes shorter than the target ranges listed in this table. All FFPE samples should be incubated at 37°C for 15 minutes to generate fragment ends suitable for library construction. Libraries prepared from FFPE samples should be analyzed using an NGS read length suitable for the final library fragment size distribution.

- 4 Prepare the appropriate volume of Fragmentation master mix by combining the reagents in [Table 15](#).

Mix well by pipetting up and down 20 times or seal the tube and vortex at high speed for 5–10 seconds. Spin briefly to remove any bubbles and keep on ice.

Table 15 Preparation of Fragmentation master mix

| Reagent | Volume for 1 reaction | Volume for 8 reactions (includes excess) | Volume for 24 reactions (includes excess) |
|------------------------------------|-----------------------|--|---|
| 5X SureSelect Fragmentation Buffer | 2 µl | 18 µl | 50 µl |
| SureSelect Fragmentation Enzyme | 1 µl | 9 µl | 25 µl |
| Total | 3 µl | 27 µl | 75 µl |

- 5 Add 3 µl of the Fragmentation master mix to each sample well containing 7 µl of input DNA.
- 6 Mix well by pipetting up and down 20 times or cap the wells and vortex at high speed for 5–10 seconds. Spin the samples briefly.
- 7 Immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in [Table 13](#).
- 8 When the program reaches the 4°C Hold step, remove the samples from the thermal cycler, add 40 µl of nuclease-free water to each sample, and place the samples on ice.

The 50-µl reactions are now ready for NGS sequencing library preparation, beginning with end repair/dA-tailing. Proceed to [“Library Preparation”](#) on page 31.

NOTE

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

2 Preparation and Fragmentation of Input DNA

Method 2: Enzymatic DNA Fragmentation



3 Library Preparation

- Step 1. Prepare the Ligation master mix 33
- Step 2. Repair and dA-Tail the DNA ends 34
- Step 3. Ligate the molecular-barcoded adaptor 36
- Step 4. Purify the sample using AMPure XP beads 37
- Step 5. Amplify the adaptor-ligated library 39
- Step 6. Purify the amplified library with AMPure XP beads 42
- Step 7. Assess quality and quantity 44

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

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

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

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



3 Library Preparation

Table 16 Reagents thawed before use in protocol

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

Step 1. Prepare the Ligation master mix

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

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

CAUTION

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

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

- 2 Prepare the appropriate volume of Ligation master mix by combining the reagents in [Table 17](#).

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

Keep at room temperature for 30–45 minutes before use on [page 36](#).

Table 17 Preparation of Ligation master mix

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

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

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

3 Library Preparation

Step 2. Repair and dA-Tail the DNA ends

Step 2. Repair and dA-Tail the DNA ends

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

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

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

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

NOTE

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

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

CAUTION

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

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

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

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

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

- 4 Add 20 μ l of the End Repair/dA-Tailing master mix to each sample well containing approximately 50 μ l of fragmented DNA. Mix by pipetting up and down 15–20 times using a pipette set to 50 μ l or cap the wells and vortex at high speed for 5–10 seconds.
- 5 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in [Table 18](#).

3 Library Preparation

Step 3. Ligate the molecular-barcoded adaptor

Step 3. Ligate the molecular-barcoded adaptor

- 1 Once the thermal cycler reaches the 4°C Hold step, transfer the samples to ice while setting up this step.
- 2 Preprogram a thermal cycler with the program in [Table 20](#) for the Ligation step. Immediately pause the program, and keep paused until samples are loaded in [step 5](#).

Table 20 Thermal cycler program for Ligation*

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

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

- 3 To each end-repaired/dA-tailed DNA sample (approximately 70 µl), add 25 µl of the Ligation master mix that was prepared on [page 33](#) and kept at room temperature. Mix by pipetting up and down at least 10 times using a pipette set to 70 µl or cap the wells and vortex at high speed for 5–10 seconds. Briefly spin the samples.
- 4 Add 5 µl of SureSelect XT HS2 Adaptor Oligo Mix (clear-capped tube) to each sample. Mix by pipetting up and down 15–20 times using a pipette set to 70 µl or cap the wells and vortex at high speed for 5–10 seconds.

NOTE

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

- 5 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in [Table 20](#).

NOTE

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

Stopping Point

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

Step 4. Purify the sample using AMPure XP beads

- 1 Verify that the AMPure XP beads were held at room temperature for at least 30 minutes before use.
- 2 Prepare 400 μ l 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 μ l of homogeneous AMPure XP beads to each DNA sample (approximately 100 μ l) in the PCR plate or strip tube. Pipette up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds to mix.
- 5 Incubate samples for 5 minutes at room temperature.
- 6 Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 to 10 minutes).
- 7 Keep the plate or strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
- 8 Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 μ l 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.

3 Library Preparation

Step 4. Purify the sample using AMPure XP beads

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

NOTE

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

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

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

15 Incubate for 2 minutes at room temperature.

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

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

NOTE

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

Step 5. Amplify the adaptor-ligated library

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

Table 21 Reagents for pre-capture PCR amplification

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

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

- 1 Determine the appropriate index pair assignment for each sample. See [Table 51](#) through [Table 58](#) in the “Reference” chapter for sequences of the 8 bp index portion of the primers used to amplify the DNA libraries in this step.

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

CAUTION

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

3 Library Preparation

Step 5. Amplify the adaptor-ligated library

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

Table 22 Pre-Capture PCR Thermal Cycler Program*

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

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

Table 23 Pre-capture PCR cycle number recommendations

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

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

CAUTION

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

- 3 Prepare the appropriate volume of pre-capture PCR reaction mix, as described in [Table 24](#), on ice. Mix well on a vortex mixer.

Table 24 Preparation of Pre-Capture PCR Reaction Mix

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

- 4 Add 11 µl of the PCR reaction mixture prepared in [Table 24](#) to each purified DNA library sample (34 µl) in the PCR plate wells.
- 5 Add 5 µl of the appropriate SureSelect XT HS2 Index Primer Pair to each reaction.
Cap the wells then vortex at high speed for 5 seconds. Spin the plate or strip tube briefly to collect the liquid and release any bubbles.
- 6 Before adding the samples to the thermal cycler, resume the thermal cycling program in [Table 22](#) to bring the temperature of the thermal block to 98°C. Once the cycler has reached 98°C, immediately place the sample plate or strip tube in the thermal block and close the lid.

CAUTION

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

3 Library Preparation

Step 6. Purify the amplified library with AMPure XP beads

Step 6. Purify the amplified library with AMPure XP beads

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

Step 6. Purify the amplified library with AMPure XP beads

17 Remove the cleared supernatant (approximately 15 μ l) 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- μ l supernatant volume at this step; transfer the maximum possible amount of supernatant for further processing.

3 Library Preparation

Step 7. Assess quality and quantity

Step 7. Assess quality and quantity

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

Table 25 Pre-capture library analysis options

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

Table 26 Pre-capture library qualification guidelines

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

- 1 Set up the instrument as instructed in the appropriate user guide (links provided in [Table 25](#)).
- 2 Prepare samples for analysis by diluting 1 μ l of each prepared library sample in 4 μ l of nuclease-free water.
- 3 Prepare the diluted samples for analysis and set up the assay as instructed in the appropriate user guide. Load the analysis assay into the instrument and complete the run.
- 4 Verify that the electropherogram shows the expected DNA fragment size peak position (see [Table 26](#) for guidelines). Sample TapeStation system electropherograms are shown for libraries prepared from sheared DNA designed for 2 \times 100 bp reads in [Figure 2](#) (high-quality DNA), [Figure 3](#) (medium-quality FFPE DNA), and [Figure 4](#) (low-quality FFPE DNA).
Electropherograms obtained using the other analysis platform options listed in [Table 25](#) are expected to show similar fragment size profiles.
- 5 Determine the concentration of the library DNA by integrating under the peak.

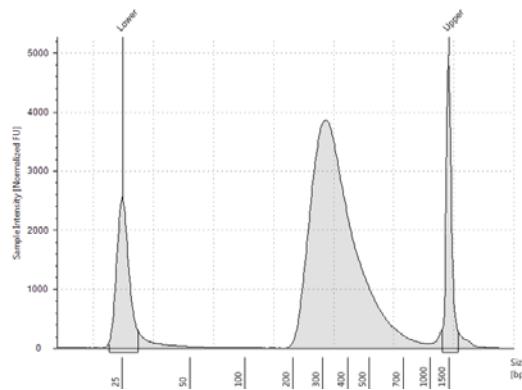


Figure 2 Pre-capture library prepared from a sheared high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

3 Library Preparation

Step 7. Assess quality and quantity

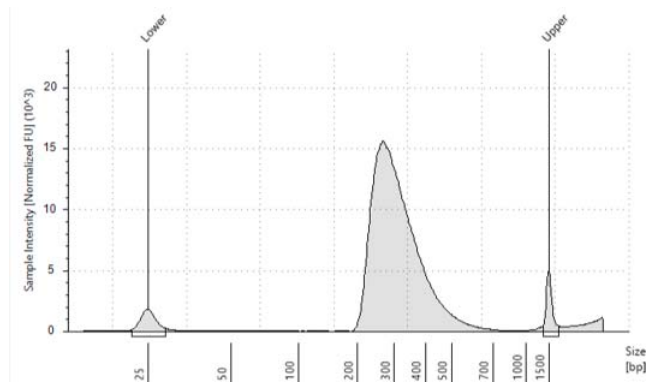


Figure 3 Pre-capture library prepared from a typical FFPE gDNA sample (fragmented by shearing) analyzed using a D1000 ScreenTape assay.

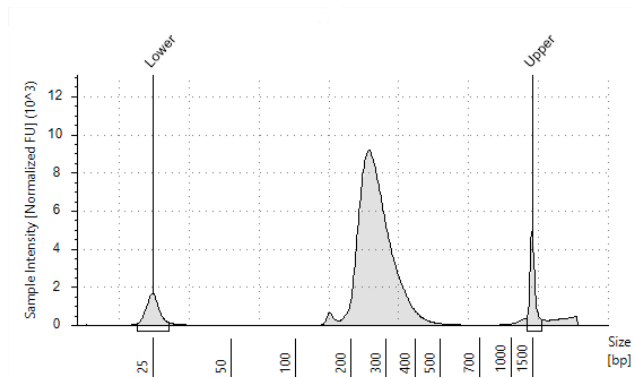


Figure 4 Pre-capture library prepared from a low-quality FFPE gDNA sample (fragmented by shearing) analyzed using a D1000 ScreenTape assay.

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

NOTE

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

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

3 Library Preparation
Step 7. Assess quality and quantity



4 Hybridization and Capture

- Step 1. Hybridize DNA libraries to the probe 50
- Step 2. Prepare streptavidin-coated magnetic beads 55
- Step 3. Capture the hybridized DNA using streptavidin-coated beads 56

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

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

CAUTION

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



4 Hybridization and Capture

Step 1. Hybridize DNA libraries to the probe

Step 1. Hybridize DNA libraries to the probe

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

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

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

Table 27 Reagents for Hybridization

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

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

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

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

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

Table 29 Hybridization program for all other probes *

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

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

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

4 Hybridization and Capture

Step 1. Hybridize DNA libraries to the probe

NOTE

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

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

- 2 Place 1000 ng of each prepared gDNA library sample into the hybridization plate or strip tube wells and then bring the final volume in each well to 12 µl using nuclease-free water. If 1000 ng DNA is not available for any of the samples, use the maximum amount available, within the 500–1000 ng range.
- 3 To each DNA library sample well, add 5 µl of SureSelect XT HS2 Blocker Mix (blue cap). Seal the wells then vortex at high speed for 5 seconds. Spin briefly to collect the liquid and release any bubbles.

CAUTION

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

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

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

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

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

Table 30 Preparation of RNase Block solution

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

NOTE

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

- 6 Prepare the Probe Hybridization Mix appropriate for your probe design size. Use Table 31 for probes ≥ 3 Mb or Table 32 for probes < 3 Mb. **Combine the listed reagents at room temperature.** Mix well by vortexing at high speed for 5 seconds then spin down briefly. Proceed immediately to step 7.

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

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

4 Hybridization and Capture

Step 1. Hybridize DNA libraries to the probe

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

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

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

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

The hybridization reaction wells now contain approximately 30 µl.

- 8 Seal the wells with fresh domed strip caps. Make sure that all wells are completely sealed. Vortex briefly, then spin the plate or strip tube briefly to remove any bubbles from the bottom of the wells. Immediately return the plate or strip tube to the thermal cycler.
- 9 Resume the thermal cycling program to allow hybridization of the prepared DNA samples to the probe.

CAUTION

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

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

Step 2. Prepare streptavidin-coated magnetic beads

The remaining hybridization capture steps use the reagents in [Table 33](#).

NOTE

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

Table 33 Reagents for Capture

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

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

NOTE

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

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

- 1 After all streptavidin bead preparation steps are complete and with the hybridization thermal cycling program in the final hold segment (see [Table 28](#) or [Table 29](#) on page 51), transfer the samples to room temperature.
- 2 Immediately transfer the entire volume (approximately 30 μ l) of each hybridization mixture to wells containing 200 μ l of washed streptavidin beads using a multichannel pipette.
Pipette up and down 5–8 times to mix then seal the wells with fresh caps.
- 3 Incubate the capture plate or strip tube on a 96-well plate mixer, mixing vigorously (at 1400–1900 rpm), for 30 minutes at room temperature.
Make sure the samples are properly mixing in the wells.
- 4 During the 30-minute incubation for capture, prewarm SureSelect Wash Buffer 2 at 70°C as described below.
 - a Place 200- μ l aliquots of Wash Buffer 2 in wells of a fresh 96-well plate or strip tubes. Aliquot 6 wells of buffer for each DNA sample in the run.
 - b Cap the wells and then incubate in the thermal cycler held at 70°C until used in [step 9](#).
- 5 When the 30-minute capture incubation period initiated in [step 3](#) is complete, spin the samples briefly to collect the liquid.
- 6 Put the plate or strip tube in a magnetic separator to collect the beads. Wait until the solution is clear, then remove and discard the supernatant.
- 7 Resuspend the beads in 200 μ l 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.

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

CAUTION

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

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

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

-
- 9 Remove the plate or strip tubes from the magnetic separator and transfer to a rack at room temperature.** Wash the beads with Wash Buffer 2, using the protocol steps below.
- a** Resuspend the beads in 200 µl of 70°C prewarmed Wash Buffer 2. Pipette up and down 15–20 times, until beads are fully resuspended.
 - b** Seal the wells with fresh caps and then vortex at high speed for 8 seconds. Spin the plate or strip tube briefly to collect the liquid without pelleting the beads.
Make sure the beads are in suspension before proceeding.
 - c** Incubate the samples for 5 minutes at 70°C on the thermal cycler with the heated lid on.
 - d** Put the plate or strip tube in the magnetic separator at room temperature.
 - e** Wait 1 minute for the solution to clear, then remove and discard the supernatant.
 - f** Repeat [step a](#) through [step e](#) five more times for a total of 6 washes.
- 10** After verifying that all wash buffer has been removed, add 25 µl of nuclease-free water to each sample well. Pipette up and down 8 times to resuspend the beads.

Keep the samples on ice until they are used on [page 62](#).

NOTE

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

4 Hybridization and Capture

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



5 Post-Capture Sample Processing for Multiplexed Sequencing

- Step 1. Amplify the captured libraries 60
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- Step 4. Pool samples for multiplexed sequencing 68
- Step 5. Prepare sequencing samples 70
- Step 6. Do the sequencing run and analyze the data 72

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



5 Post-Capture Sample Processing for Multiplexed Sequencing

Step 1. Amplify the captured libraries

Step 1. Amplify the captured libraries

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

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

Table 34 Reagents for post-capture PCR amplification

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

Prepare one amplification reaction for each DNA library.

CAUTION

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

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

Table 35 Post-capture PCR Thermal Cycler Program *

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

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

Table 36 Post-capture PCR cycle number recommendations

| Probe Capture Library Size | Cycles |
|--|--------------|
| Probes <0.2 Mb | 16 cycles |
| Probes 0.2–3 Mb | 12–16 cycles |
| Probes 3–5 Mb | 11–12 cycles |
| Probes >5 Mb (including Human All Exon probes) | 10–11 cycles |

5 Post-Capture Sample Processing for Multiplexed Sequencing

Step 1. Amplify the captured libraries

- 2 Prepare the appropriate volume of PCR reaction mix, as described in [Table 37](#), on ice. Mix well on a vortex mixer.

Table 37 Preparation of post-capture PCR reaction mix

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

- 3 Add 25 μ l of the PCR reaction mix prepared in [Table 37](#) to each sample well containing 25 μ l of bead-bound target-enriched DNA (prepared on [page 57](#) and held on ice).
- 4 Mix the PCR reactions well by pipetting up and down until the bead suspension is homogeneous. Avoid splashing samples onto well walls; do not spin the samples at this step.
- 5 Place the plate or strip tube in the thermal cycler and resume the thermal cycling program in [Table 35](#).
- 6 When the PCR amplification program is complete, spin the plate or strip tube briefly. Remove the streptavidin-coated beads by placing the plate or strip tube on the magnetic stand at room temperature. Wait 2 minutes for the solution to clear, then **remove each supernatant (approximately 50 μ l) to wells of a fresh plate or strip tube.**
The beads can be discarded at this time.

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

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

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

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

- 14** Seal the sample wells with strip caps, then mix well on a vortex mixer and briefly spin to collect the liquid without pelleting the beads.
- 15** Incubate for 2 minutes at room temperature.
- 16** Put the plate or strip tube in the magnetic stand and leave for 2 minutes or until the solution is clear.
- 17** Remove the cleared supernatant (approximately 25 μ l) to a fresh well. You can discard the beads at this time.

Step 3. Assess sequencing library DNA quantity and quality

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

Table 38 Post-capture library analysis options

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

Table 39 Post-capture library qualification guidelines

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

Step 3. Assess sequencing library DNA quantity and quality

- 1 Set up the instrument as instructed in the appropriate user guide (links provided in [Table 38](#)).
- 2 Prepare the samples for analysis and set up the assay as instructed in the appropriate user guide. Load the analysis assay into the instrument and complete the run.
- 3 Verify that the electropherogram shows the expected DNA fragment size peak position (see [Table 39](#) for guidelines). Sample TapeStation system electropherograms are shown for libraries prepared from sheared DNA designed for 2 ×100 bp reads in [Figure 5](#) (high-quality input DNA), [Figure 6](#) (medium-quality FFPE input DNA), and [Figure 7](#) (low-quality FFPE input DNA).

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

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

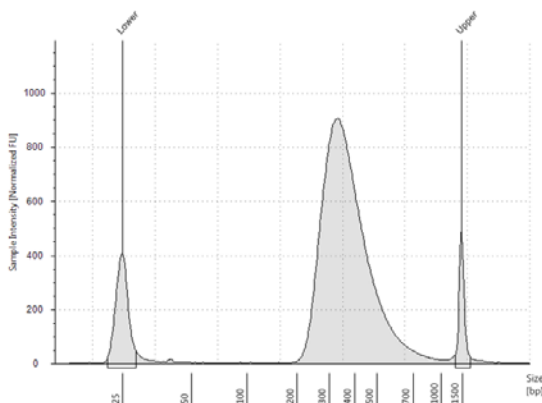


Figure 5 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

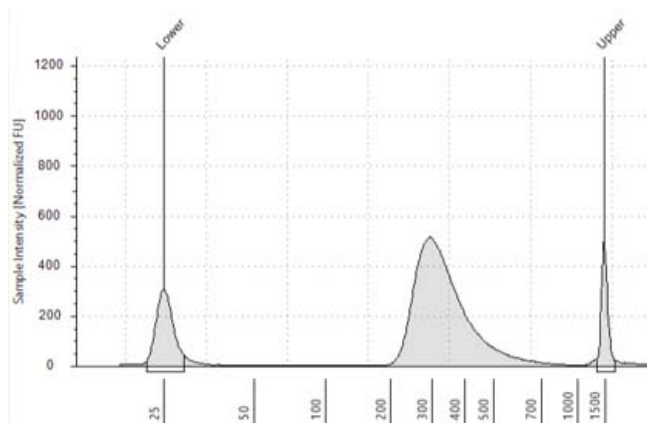


Figure 6 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

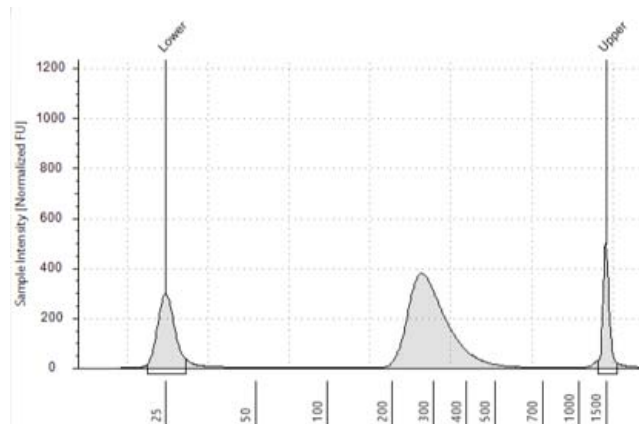


Figure 7 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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

Step 4. Pool samples for multiplexed sequencing

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

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

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

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

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

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

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

$\#$ is the number of indexes, and

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

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

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

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

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

Step 5. Prepare sequencing samples

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

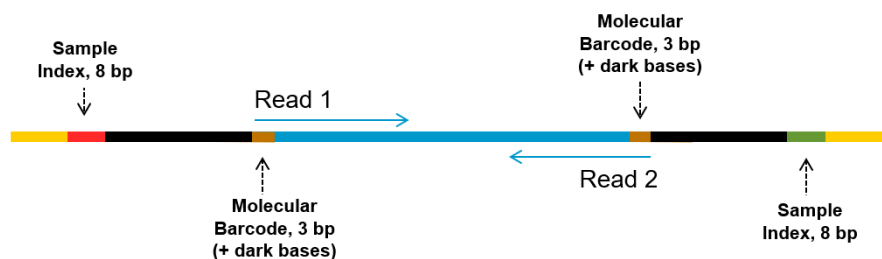


Figure 8 Content of SureSelect XT HS2 sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), unique dual sample indexes (red and green), duplex molecular barcodes (brown) and the library PCR primers (yellow).

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

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

The optimal seeding concentration for SureSelect XT HS2 target-enriched libraries varies according to sequencing platform, run type, and Illumina kit version. See [Table 41](#) for guidelines. Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in [Table 41](#).

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

Table 41 Illumina Kit Configuration Selection Guidelines

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

Step 6. Do the sequencing run and analyze the data

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

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

HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

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

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

Table 42 Run settings

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

MiSeq platform sequencing run setup guidelines

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

Set up a custom Sample Sheet:

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

Step 6. Do the sequencing run and analyze the data

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

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

Sample Sheet Wizard - Workflow Parameters

FASTQ Only Run Settings

Reagent Cartridge Barcode* MSS871368-300V2

Library Prep Workflow **TruSeq Nano DNA**

Index Adapters **TruSeq DNA CD Indexes (96 Indexes)**

Index Reads 0 (None) 1 (Single) 2 (Dual)

Experiment Name

Investigator Name

Description

Date 1/22/2018

Read Type Paired End Single Read

Cycles Read 1 100

Cycles Read 2 100

* - required field

FASTQ Only Workflow-Specific Settings

Custom Primer for Read 1

Custom Primer for Index

Custom Primer for Read 2

Reverse Complement

Use Adapter Trimming

Use Adapter Trimming Read 2

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

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

EM Illumina Experiment Manager
Illumina Experiment Manager

Sample Sheet Wizard - Sample Selection

Samples to include in sample sheet

| 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 | ATTTCAGAA | D501 | TATAGCCT | | |
| 6 | 6 | Plate1 | A06 | D706 | GAATTCGT | D501 | TATAGCCT | | |

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

Step 6. Do the sequencing run and analyze the data

Editing the Sample Sheet to include SureSelect XT HS2 dual indexes

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

| [Header] | | | | | | | | |
|-------------------|----------------------|--------------|-------------|-------------|----------|-------------|----------|--------|
| Investigator Name | NN | | | | | | | |
| Project Name | Sequencing Project A | | | | | | | |
| Experiment Name | Experiment 1 | | | | | | | |
| Date | 3/20/2013 | | | | | | | |
| Workflow | GenerateFASTQ | | | | | | | |
| Assay | SureSelect XT HS V2 | | | | | | | |
| Chemistry | SureSelect XT HS V2 | | | | | | | |
| [Reads] | | | | | | | | |
| | 100 | | | | | | | |
| | 100 | | | | | | | |
| [Settings] | | | | | | | | |
| OnlyGenerateFASTQ | 1 | | | | | | | |
| [Data] | | | | | | | | |
| Sample_ID | Sample_Name | Sample_Plate | Sample_Well | I7_Index_ID | index | I5_Index_ID | index2 | Sample |
| Sample 1 | Sample1 | Plate1 | A01 | 01 | CAAGGTGA | 01 | ATGGTTAG | |
| Sample 2 | Sample2 | Plate1 | A02 | 02 | TAGACCAA | 02 | CAAGGTGA | |
| Sample 3 | Sample3 | Plate1 | A03 | 03 | AGTCGCGA | 03 | TAGACCAA | |

Figure 9 Sample sheet for SureSelect XT HS2 library sequencing

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

Sequence analysis resources

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

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

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

NOTE

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

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

Sequence analysis resources



6 Appendix: Using FFPE-derived DNA Samples

| | |
|--|----|
| Protocol modifications for FFPE Samples | 80 |
| Methods for FFPE Sample Qualification | 80 |
| Sequencing Output Recommendations for FFPE Samples | 81 |

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



Protocol modifications for FFPE Samples

Protocol modifications that should be applied to FFPE samples are summarized in [Table 43](#).

Table 43 Summary of protocol modifications for FFPE samples

| Workflow Step and page | Parameter | Condition for non-FFPE Samples | Condition for FFPE Samples |
|---|--|---|--|
| gDNA Sample Preparation page 22 | Qualification of DNA Integrity | Not required | Required |
| DNA input for Library Preparation page 22 | Input amount and means of quantification | 10 ng to 200 ng, quantified by Qubit assay | Based on determined DNA integrity (see Table 9 on page 23 and Table 10 on page 24) |
| DNA Shearing page 26 | Mode of DNA Shearing | 2 × 120 seconds (for 2 × 100 reads) 2 × 60 seconds (for 2 × 150 reads) | 240 seconds (continuous, for all read lengths) |
| Pre-capture PCR page 40 | Cycle number | 8–11 | 11–14 |
| Sequencing page 81 | Output augmentation | Per project requirements | 1× to 10× based on determined DNA integrity (see Table 44 and Table 45 on page 81) |

Methods for FFPE Sample Qualification

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

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include the precise quantity of amplifiable DNA in the sample to allow direct normalization of input DNA amount and a $\Delta\Delta Cq$ DNA integrity score used to design other protocol modifications.

The Agilent TapeStation instrument, combined with the Genomic DNA ScreenTape assay, provides an automated electrophoresis method for determination of a DNA Integrity Number (DIN) score used to estimate amount of input DNA required for sample normalization and to design other protocol modifications.

Sequencing Output Recommendations for FFPE Samples

After determining the amount of sequencing output required for intact DNA samples to meet the goals of your project, use the guidelines below to determine the amount of extra sequencing output required for FFPE DNA samples.

Samples qualified using $\Delta\Delta Cq$: For samples qualified based on the $\Delta\Delta Cq$ DNA integrity score, use the guidelines in [Table 44](#). For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with $\Delta\Delta Cq$ score of 1 requires 200–400 Mb of sequencing output to achieve the same coverage.

Table 44 Recommended sequencing augmentation for FFPE-derived DNA samples

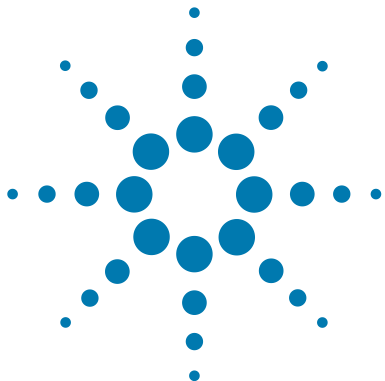
| $\Delta\Delta Cq$ value | Recommended fold increase for FFPE-derived sample |
|-------------------------|---|
| <0.5 | No extra sequencing output |
| between 0.5 and 2 | Increase sequencing allocation by 2× to 4× |
| >2 | Increase sequencing allocation by 5× to 10× or more |

Samples qualified using DIN: For samples qualified based on the Genomic DNA ScreenTape assay DIN integrity score, use the guidelines in [Table 45](#). For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with DIN score of 4 requires approximately 200–400 Mb of sequencing output to achieve the same coverage.

Table 45 Recommended sequencing augmentation for FFPE-derived DNA samples

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

6 Appendix: Using FFPE-derived DNA Samples
Sequencing Output Recommendations for FFPE Samples



7 Reference

| | |
|---|-----|
| Kit Contents | 84 |
| SureSelect XT HS2 Index Primer Pair Information | 86 |
| Troubleshooting Guide | 98 |
| Quick Reference Protocol | 102 |

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



Kit Contents

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

Table 46 Component Kits

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

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

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

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

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

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

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

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

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

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

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

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

SureSelect XT HS2 Index Primer Pair Information

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

NOTE

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

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

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

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

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

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

7 Reference

SureSelect XT HS2 Index Primer Pair Information

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

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

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

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

7 Reference

SureSelect XT HS2 Index Primer Pair Information

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

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

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

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

7 Reference

SureSelect XT HS2 Index Primer Pair Information

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

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

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

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

7 Reference

SureSelect XT HS2 Index Primer Pair Information

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

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

Index Primer Pair Strip Tube and Plate Maps

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

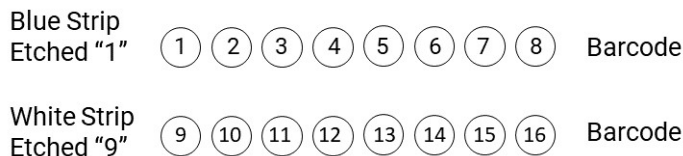


Figure 10 Map of the SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) strip tubes provided with 16 reaction kits

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

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

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

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

CAUTION

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

7 Reference

Index Primer Pair Strip Tube and Plate Maps

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

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

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

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

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

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

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

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

Troubleshooting Guide

If recovery of gDNA from samples is low

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

If yield of pre-capture libraries is low

- ✓ The library preparation protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance of the highly viscous buffer and enzyme solutions used in the protocol. Be sure to adhere to all instructions when setting up the reactions.
- ✓ Ensure that the ligation master mix (see [page 33](#)) is kept at room temperature for 30–45 minutes before use.
- ✓ PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the pre-capture PCR cycle number by 1 to 2 cycles. If a high molecular weight peak (>500 bp) is observed in the electropherogram for a sample with low yield, the DNA may be overamplified. Repeat library preparation for the sample, decreasing the pre-capture PCR cycle number by 1 to 3 cycles.
- ✓ DNA isolated from degraded samples, including FFPE tissue samples, may be over-fragmented or have modifications that adversely affect library preparation processes. Use the Agilent NGS FFPE QC Kit to determine the precise quantity of amplifiable DNA in the sample and allow direct normalization of input DNA amount.

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

If solids observed in the End Repair-A Tailing Buffer

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

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

- ✓ Shearing may not be optimal. For intact, high-quality DNA samples, ensure that shearing is completed using the two-round shearing protocol provided, including all spinning and vortexing steps.
- ✓ Any bubbles present on the microTUBE filament may disrupt complete shearing. Spin the microTUBE for 30 seconds before the first round of shearing to ensure that any bubbles are released.

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

- ✓ FFPE DNA pre-capture libraries may have a smaller fragment size distribution due to the presence of DNA fragments in the input DNA that are smaller than the target DNA shear size.
- ✓ DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color and verify that you are using the bead volume recommended for pre-capture purification on [page 42](#).

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

- ✓ The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to the example shown in [Figure 4](#) on page 46. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield of pre-capture libraries. If excessive adaptor-dimers are observed, verify that the adaptor ligation protocol is being performed as directed on [page 36](#). In particular, ensure that the Ligation master mix is mixed with the sample prior to adding the Adaptor Oligo Mix to the mixture. Do not add the Ligation master mix and the Adaptor Oligo Mix to the sample in a single step.
- ✓ For whole-genome sequencing (not specifically supported by this protocol), samples with an adaptor-dimer peak must be subjected to an additional round of SPRI-purification. To complete, first dilute the sample to 50 µl with nuclease free water, then follow the SPRI purification procedure on [page 42](#).

If yield of post-capture libraries is low

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

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

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

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

- ✓ Stringency of post-hybridization washes may have been lower than required. Complete the wash steps as directed, paying special attention to the details of SureSelect Wash Buffer 2 washes listed below:
 - SureSelect Wash Buffer 2 is pre-warmed to 70°C (see [page 56](#))
 - Samples are maintained at 70°C during washes (see [page 57](#))
 - Bead suspensions are mixed thoroughly during washes by pipetting up and down **and** vortexing (see [page 57](#))
- ✓ Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a vortex and plate spinner or centrifuge in close proximity to thermal cycler to retain the 65°C sample temperature during mixing and transfer steps ([step 8](#) to [step 9](#) on [page 54](#)).

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

- ✓ High AT-dropout may indicate that hybridization conditions are too stringent to obtain the desired level of coverage for AT-rich targets.
 - For libraries target-enriched using the SureSelect XT HS Human All Exon V8 Probe and the hybridization program in [Table 28](#) on [page 51](#) (including segment with one-hour incubation at 65°C), repeat target enrichment using the hybridization program in [Table 29](#) on [page 51](#) (without the one-hour incubation at 65°C segment).
 - For other probes, repeat target enrichment at lower stringency using a modified thermal cycler program for hybridization, reducing the hybridization temperature in segments 4 and 5 from 65°C to 62.5°C or 60°C (see [Table 29](#) on [page 51](#)).
- ✓ Redesign custom target enrichment probes designed for the XT platform employing SureDesign's XT HS-boosting parameters during design.

Quick Reference Protocol

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

| Step | Summary of Conditions |
|--|--|
| Library Prep | |
| Prepare, qualify, and fragment DNA samples | Prepare 10–200 ng gDNA in Low TE (50 µl for Covaris/7 µl for enzymatic fragmentation) For FFPE DNA, qualify integrity and adjust input amount as directed on page 23 and page 24 Mechanically shear DNA using Covaris with shearing conditions on page 25 OR enzymatically fragment DNA using SureSelect Enzymatic Fragmentation Kit with protocol on page 28 (50 µl final volume) |
| Prepare Ligation master mix | Per reaction: 23 µl Ligation Buffer + 2 µl T4 DNA Ligase Keep at room temperature 30–45 min before use |
| Prepare End-Repair/dA-Tailing master mix | Per reaction: 16 µl End Repair-A Tailing Buffer + 4 µl End Repair-A Tailing Enzyme Mix Keep on ice |
| End-Repair and dA-Tail the DNA fragments | 50 µl DNA fragments + 20 µl End Repair/dA-Tailing master mix Incubate in thermal cycler: 15 min @ 20°C, 15 min @ 72°C, Hold @ 4°C |
| Ligate adaptor | 70 µl DNA sample + 25 µl Ligation master mix + 5 µl SureSelect XT HS2 Adaptor Oligo Mix Incubate in thermal cycler: 30 min @ 20°C, Hold @ 4°C |
| Purify DNA | 100 µl DNA sample + 80 µl AMPure XP bead suspension Elute DNA in 35 µl nuclease-free H ₂ O, removing 34 µl to fresh well |
| Prepare PCR master mix | Per reaction: 10 µl 5× Herculase II Reaction Buffer with dNTPs + 1 µl Herculase II Fusion DNA Polymerase Keep on ice |
| Amplify the purified DNA | 34 µl purified DNA + 11 µl PCR master mix + 5 µl assigned SureSelect XT HS2 Index Primer Pair Amplify in thermal cycler using program on page 40 |
| Purify amplified DNA | 50 µl amplified DNA + 50 µl AMPure XP bead suspension Elute DNA in 15 µl nuclease-free H ₂ O |
| Quantify and qualify DNA | Analyze quantity and quality using TapeStation, Bioanalyzer, or Fragment Analyzer System |

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

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In This Book

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

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