SureSelect Cancer CGP Assay

User Guide

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

Version D0, April 2024

Notices

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Acknowledgments

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In This Guide…

This guide provides instructions for the SureSelect Cancer CGP Assay, a targeted next-generation sequencing (NGS) solution for interrogation of genomic and transcriptomic features of relevance in solid tumors. Support is also provided for SureSelect Cancer Tumor-Specific and Custom Panel Assays, performed using minor protocol modifications.

What's New in Version D0

- Support for SureSelect Cancer Custom Assays (see **page 8** and **"Appendix 3: SureSelect Cancer Custom Panel Assays"** on page 70)
- Access information for the SureSelect XT HS2 Index Sequence Resource Excel spreadsheet (see **page 79**)

What's New in Version C0

- Support for Agilent's Alissa Reporter analysis software v1.3 (see **page 8**, **page 9**, **page 10** **page 11**, **page 48**, and **page 51** to **page 58**).
- Update to Cancer CGP DNA Assay MSI target and coverage descriptions (see **page 8** and **page 58**)
- Updates to Magnis automation RNA assay and Magnis firmware v1.4 availability information (see **page 59** and **page 60**)
- Updates to Bravo automation information for NGS Bravo Option B+ users (see footnote to **Table 47** on page 61)

What's New in Version B0

- Support for new SureSelect Cancer Tumor-Specific Assays (see **page 8** and **"Appendix 2: SureSelect Cancer Tumor-Specific Assays"** on page 67)
- Update to Cancer CGP Assay CNV target description to 32 loci (see **page 8**)
- Correction of typographical errors in *Note* on **page 25**
- Updates to downstream sequencing support information (see **page 51** to **page 52**)
- Updates to **Notices** section

Content

1
Before You Begin

Introduction to the SureSelect Cancer CGP Assay

The SureSelect Cancer Comprehensive Genomic Profiling (CGP) Assay is a targeted next-generation sequencing (NGS) solution that enables interrogation of genomic and transcriptomic regions of relevance in solid tumors for a variety of features including those listed below.

- **DNA SNVs (single nucleotide variations) and Indels (short insertions and deletions):** The DNA Assay probe design includes full exonic coverage of 679 cancer-associated genes, allowing comprehensive cancer gene variant calling against the reference genome.
- **DNA CNVs (copy number variations):** The DNA Assay probe design includes optimal target sequence placement at 32 loci for detection of DNA amplification and deletion at key loci.
- **DNA Translocations:** The DNA Assay probe design includes intronic coverage in 12 genes to enable translocation detection at key loci.
- **Tumor Mutational Burden (TMB):** Coverage of >1.6 Mb of coding sequence enables quantification of TMB in DNA samples.
- **Microsatellite Instability (MSI):** The DNA assay probe targets 288 sites available for microsatellite instability (MSI) determinations, with coverage for typical samples in the range of 250-280 sites, enabling quantification of MSI in DNA samples.
- **RNA fusions and exon-skipping RNA splice variants**: The RNA Assay probe design enables detection of RNA fusions in 80 key genes (regardless of partner) and of important splice variants *EGFRvIII* and *MET Exon14-skipping*.

The assay includes SureSelect XT HS2 reagents for preparation of target-enriched NGS libraries from gDNA and total RNA samples. The workflow is summarized in Figure 1.

Once sequencing data is collected for the assay samples, Agilent offers Alissa Reporter analysis applications tailored to the SureSelect Cancer Comprehensive Genomic Profiling (CGP) Assays. See page 53 for more information. Alternative NGS analysis software tools can also be used for variant discovery.

The protocols provided in this publication may also be used to interrogate gDNA samples using Agilent's SureSelect Cancer Tumor-Specific Assays (see page 67) or SureSelect Cancer Custom Panel Assays (see page 70).

Sample requirements

The SureSelect Cancer CGP Assay supports analysis of DNA and RNA samples isolated from fresh or fresh-frozen samples or extracted from formalin-fixed, paraffin-embedded (FFPE) tissues. The assay is optimized for sample input amounts of 50 ng genomic DNA or 50 ng total RNA. For low-quality FFPE samples, assay performance may be improved by increasing the amount of DNA or RNA input to up to 200 ng. Use of 10–200 ng DNA or RNA input is supported by the SureSelect XT HS2 system; however, use of input <50 ng for the SureSelect Cancer CGP Assay may lead to lower target coverage and reduced detection of low-frequency variants. See *Troubleshooting* on page 86 for more information on use of low input (<50 ng) samples.

FFPE samples should be isolated from a minimum of 3 tissue block sections of 5 µm each and containing ≥15% tumor content. Agilent has not validated the SureSelect Cancer CGP Assay using liquid biopsy or needle aspiration samples. See *Troubleshooting* on page 88 for more information on use of unsupported sample types.

Consult the selected analysis software guidelines for any additional sample requirements. The assay supports tumor-normal paired analysis using matched or unmatched reference DNA where variant analysis may require specific types or numbers of reference samples. For example, CNV analysis algorithms typically require co-processing of matched or unmatched reference DNA samples without copy number aberrations in the regions of interest. Reference DNA samples may be isolated from normal FFPE tissue blocks, fresh-frozen tissues or cell lines. Agilent's OneSeq Human Reference DNA is recommended for use as an unmatched reference DNA sample.

Sample requirements for Agilent's Alissa Reporter Cancer CGP applications

For analysis using Alissa Reporter, some types of variant analysis require co-processing of the tumor (*Target*) sample and a matched/unmatched normal (*Reference*) sample.

SNV/Indel analysis in Alissa Reporter can be completed in Tumor-normal or Tumor-only mode. Use of a matched/unmatched normal *Reference* sample is required for analysis in Tumor-normal mode, where the *Reference* sample is used to help filter germline variants. Using a matched reference sample for Tumor-normal mode analysis is recommended since detection of somatic variants can be less sensitive when an unmatched reference is used. When no *Reference* sample is available, analysis is performed in Tumor-only mode with reduced germline variant filtration.

CNV analysis in Alissa Reporter always requires co-processing of a matched/unmatched *Reference* sample with each tumor *Target* sample.

For best results, process the *Target* and *Reference* samples to be co-analyzed in Alissa Reporter in the same SureSelect run and in the same sequencing run in order to minimize any processing-based variance. The software allows analysis using pre-established unmatched reference sample data from a prior run but potential bias due to batch differences may negatively impact the accuracy of calling.

For variant calling on the X and Y chromosomes, it is important to use *Target* and *Reference* samples of the same sex.

Overview of the Workflow

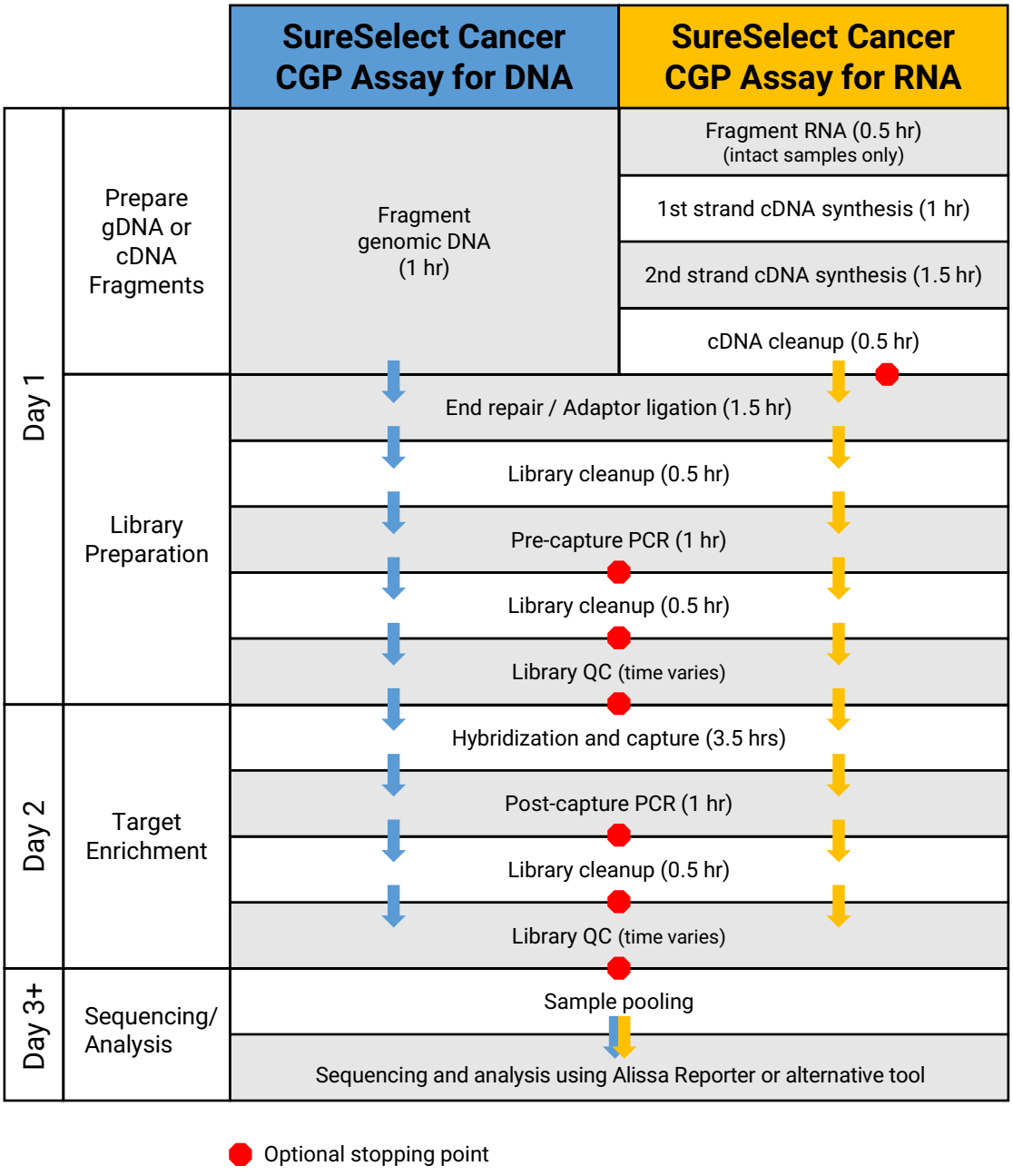

Figure 1 SureSelect Cancer CGP assay workflow. DNA and RNA samples are processed in separate reactions throughout the NGS library preparation and target enrichment steps, but can be processed in parallel beginning with the end repair/adaptor ligation workflow segment and can be sequenced and analyzed together. See page 16 for synchronization guidelines. The provided time estimates are for processing up to 16 reactions per run; your results may vary.

SureSelect Cancer CGP Assay Components

The SureSelect Cancer CGP Assay requires the components listed below:

- SureSelect Cancer CGP Assay Probes (DNA and RNA assay probes in separate vials)
- Library preparation and hybridization/capture reagents using SureSelect XT HS2 chemistry
- Optional: Alissa Reporter analysis software (Agilent p/n G5393AA for use of Alissa Cancer CGP DNA assay, p/n G5394AA for use of Alissa Cancer CGP RNA assay, or Agilent p/n M5711AA for use of both DNA and RNA assays). Please email informatics_support@agilent.com or contact your local representative for software access information.

Table 1 shows the SureSelect Cancer CGP Assay Kit formats available for non-automated sample processing. Kits for automated processing are described in Table 44 on page 59.

See Table 2 through Table 4 for additional materials required to complete the assay protocols.

Table 1 Ordering information for SureSelect Cancer CGP Assay components

Description	Agilent Part Number	Reagent Modules Included*					
		Probe(s)	DNA Library Prep + Hyb Reagents	RNA Library Prep + Hyb Reagents	Capture Beads, Purification Beads	Enzymatic DNA Fragmentation	Reference DNA/ Control RNA
Complete Starter Kit for DNA & RNA Assays (16 Samples for each assay)							
SureSelect Cancer CGP Assay Starter Kit DNA & RNA, 16 Samples Each (32 Hyb)	G9965A	✓DNA & RNA†	✓ (Index 1-16)	✓ (Index 17-32)	✓	✓	✓
DNA & RNA Assay Kit (96 Samples for each assay)							
SureSelect Cancer CGP Assay DNA & RNA Kit, 96 Samples Each (192 Hyb)	G9966A	✓DNA & RNA†	✓ (Index 1-96)	✓ (Index 97-192)	✓	– (see Table 3 on page 13)	– (see Table 3 on page 13)
DNA Assay Kits (16 Samples or 96 Samples)							
SureSelect Cancer CGP Assay DNA Kit, 16 Samples	G9967A	✓DNA†	✓ (Index 1-16)	× (not required)	✓	– (see Table 3 on page 13)	– (see Table 3 on page 13)
SureSelect Cancer CGP Assay DNA Kit, 96 Samples	G9967B	✓DNA†	✓ (Index 1-96)	× (not required)	✓	– (see Table 3 on page 13)	– (see Table 3 on page 13)
RNA Assay Kits (16 Samples or 96 Samples)							
SureSelect Cancer CGP Assay RNA Kit, 16 Samples	G9968A	✓RNA†	× (not required)	✓ (Index 17-32)	✓	× (not required)	– (see Table 3 on page 13)
SureSelect Cancer CGP Assay RNA Kit, 96 Samples	G9968B	✓RNA†	× (not required)	✓ (Index 97-192)	✓	× (not required)	– (see Table 3 on page 13)

* See "Reagent Kit Contents" on page 75 through page 78 for a complete list of Reagent Kit components provided with each product.

† The SureSelect Cancer CGP Assay Probes may also be purchased separately. See Table 59 on page 77 for part number information.

Additional Materials Required

Use the tables below to select the additional materials required to complete the SureSelect Cancer CGP Assay. Table 2 lists the materials needed for all workflows, while Table 3 and Table 4 list additional materials needed for specific sample types and protocol step options.

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 2 Required Equipment and Reagents--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	Consult the thermal cycler manufacturer's recommendations
Nucleic acid analysis system (instrument and consumables)	Select one system from Table 4 on page 14
Small-volume spectrophotometer	NanoDrop 2000, Thermo Fisher Scientific p/n ND-2000 or equivalent
Qubit Fluorometer	Thermo Fisher Scientific p/n Q33238
Qubit Assay Tubes	Thermo Fisher Scientific p/n Q32856
Qubit BR dsDNA Assay Kit 100 assays 500 assays	Thermo Fisher Scientific p/n Q32850 p/n Q32853
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930
1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276
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 and single channel pipettes	Rainin Pipet-Lite Multi Pipette or equivalent
Sterile, nuclease-free aerosol barrier pipette tips, vortex mixer, ice bucket, and powder-free gloves	General laboratory supplier

* 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 3 Additional Required Materials based on Sample Type/Fragmentation Method

Description	Vendor and Part Number	Usage Notes
Required for DNA assays (not required for RNA-only assays)		
FFPE gDNA purification system, for example: QIAamp DNA FFPE Tissue Kit, 50 Samples Deparaffinization Solution	QIAGEN p/n 56404 p/n 19093	Recommended system for FFPE gDNA sample purification.
FFPE DNA integrity assessment system: Agilent NGS FFPE QC Kit 16 reactions 96 reactions OR TapeStation Genomic DNA Analysis Consumables: Genomic DNA ScreenTape Genomic DNA Reagents	 Agilent p/n G9700A p/n G9700B Agilent p/n 5067-5365 p/n 5067-5366	Recommended systems for FFPE gDNA qualification prior to library preparation.
High-quality gDNA purification system, for example: QIAamp DNA Mini Kit 50 Samples 250 Samples	 QIAGEN p/n 51304 p/n 51306	Recommended system for purification of intact gDNA.
OneSeq Human Reference DNA, Female	Agilent p/n 5190-8850	Control and unmatched reference DNA
SureSelect Enzymatic Fragmentation Kit	Agilent p/n 5191-4080 (96 reactions)	Not required for workflows using mechanical (Covaris-mediated) DNA shearing.
Mechanical DNA fragmentation system: Covaris Sample Preparation System Covaris microTUBE sample holders	 Covaris model E220 Covaris p/n 520045	Not required for workflows using enzymatic DNA fragmentation. Additional Covaris instrument models and sample holders may be used after optimization of shearing conditions.
Required for RNA assays (not required for DNA-only assays)		
FFPE RNA purification system, for example: RNeasy FFPE Kit, 50 Samples	QIAGEN p/n 73504	Recommended system for FFPE RNA sample purification.
FFPE RNA integrity analysis system: 4200/4150 TapeStation with RNA ScreenTape/High-Sensitivity RNA ScreenTape OR 2100 Bioanalyzer with RNA 6000 Pico/Nano Kit OR 5200/5300/5400 Fragment Analyzer with RNA/HS RNA Kit	See Table 4 on page 14 for ordering information	Select the RNA analysis consumables designed for the qualification system used in your laboratory and appropriate for the RNA concentrations of your samples.
QPCR Human Reference Total RNA	Agilent p/n 750500	Control RNA

Table 4 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
RNA ScreenTape	p/n 5067-5576
RNA ScreenTape Sample Buffer	p/n 5067-5577
RNA ScreenTape Ladder	p/n 5067-5578
High Sensitivity RNA ScreenTape	p/n 5067-5579
High Sensitivity RNA ScreenTape Sample Buffer	p/n 5067-5580
High Sensitivity RNA ScreenTape Ladder	p/n 5067-5581
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
RNA 6000 Pico Kit	p/n 5067-1513
RNA 6000 Nano Kit	p/n 5067-1511
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
RNA Kit (15NT)	p/n DNF-473-0500
HS RNA Kit (15NT)	p/n DNF-474-0500

Procedural and Safety Notes

- Use best-practices to prevent PCR product and nuclease contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow.
  2. Maintain clean work areas. Clean the surfaces that pose the highest risk of contamination daily using a 10% bleach solution, or equivalent.
  3. Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
  4. Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.
- Several reagent solutions used in the SureSelect Cancer CGP Assay protocols are highly viscous. Make sure to follow the mixing instructions provided in the protocols.

- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of caps. Cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler and from other procedural steps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during thermal cycler incubation steps.
- Possible stopping points, where samples may be stored at 4°C or –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.
- In general, follow Biosafety Level 1 (BSL1) safety rules.

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

2
RNA-Specific Workflow Steps

This section describes the steps to prepare fragmented input RNA and the steps to convert the RNA fragments to strand-specific cDNA prior to sequencing library preparation. The protocols include conditions for FFPE-derived RNA samples (see page 17) and intact RNA from fresh or fresh-frozen samples (see page 18).

FFPE-derived RNA samples are already sufficiently fragmented for library preparation, while the intact RNA samples are chemically-fragmented in this step. The protocol produces cDNA fragments for the SureSelect Cancer CGP RNA Assay suitable for 2 x 150 read length NGS.

NOTE For FFPE RNA samples, initial RNA fragment size may impact the size distribution in the final cDNA library, with some library fragments shorter than 150 bp.

Guidelines for simultaneous DNA & RNA workflows

If you are preparing DNA libraries (only), proceed directly to page 23.

If you are preparing both DNA and RNA libraries in the same run, review both the RNA-specific steps in this section and the DNA-specific steps on page 23 through page 28 before you begin. Once both input gDNA and total RNA samples have been prepared and qualified, the DNA & RNA assay workflows can be synchronized by starting with the RNA-specific workflow steps (page 17 to page 21) where RNA samples are processed to purified cDNA and stored as directed on page 22 while DNA samples are fragmented.

This workflow segment uses the components listed in Table 5. Remove the listed reagents from cold storage, and prepare as directed before use (refer to the *Where Used* column).

Table 5 Reagents thawed before use in protocol

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect cDNA Module (Pre PCR), –20°C	2X Priming Buffer (tube with purple cap)	Thaw on ice then keep on ice, vortex to mix	page 19
	First Strand Master Mix (amber tube with amber cap)*	Thaw on ice for 30 minutes then keep on ice, vortex to mix	page 19
	Second Strand Enzyme Mix (tube with blue cap or bottle)	Thaw on ice then keep on ice, vortex to mix	page 20
	Second Strand Oligo Mix (tube with yellow cap)	Thaw on ice then keep on ice, vortex to mix	page 20
+4°C	SureSelect RNA AMPure XP Beads	Equilibrate at room temperature for at least 30 minutes before use, vortex to mix	page 21

* The First Strand Master Mix contains actinomycin D and is provided ready-to-use. Keep the reagent in the supplied amber vial to protect the contents from exposure to light.

Step 1. Prepare and qualify RNA samples

FFPE RNA samples

The instructions in this section are for FFPE-derived RNA samples. For intact (non-FFPE) RNA samples, instead follow the instructions on page 18.

Samples are obtained from tissue resection (tissue curls or sections on slide), with use of a minimum of 3 sections of 5 µm each and with ≥15% tumor content (measured by haemotoxylin & eosin staining) recommended.

1 Prepare total RNA from each FFPE sample in the run. The optimized library preparation protocol uses 50 ng of FFPE total RNA in a 10 µL volume of nuclease-free water. The assay may be performed using up to 200 ng input RNA.

NOTE The SureSelect XT HS2 RNA system supports use of 10–200 ng RNA input. Use of <50 ng RNA for the SureSelect Cancer CGP Assay may reduce yield and target coverage.

2 Use a small-volume spectrophotometer to determine the RNA concentration and the 260/280 and 260/230 absorbance ratio values for the sample. High-quality RNA samples are indicated by values of approximately 1.8 to 2.0 for both ratios.

3 Examine the starting RNA size distribution in the sample using one of the RNA qualification systems described in Table 6. Select the specific assay appropriate for your sample based on the RNA concentration determined in step 2.

Table 6 Agilent RNA qualification platforms

Analysis Instrument	RNA Qualification Assay	Analysis Mode
4200/4150 TapeStation	RNA Screen Tape or High Sensitivity RNA Screen Tape	Region analysis using TapeStation Analysis Software
2100 Bioanalyzer	RNA 6000 PicoChip or NanoChip	Smear/Region analysis using 2100 Expert Software
5200 Fragment Analyzer	RNA Kit (15NT) or HS RNA Kit (15NT)	Analysis using ProSize Data Analysis Software

NOTE Grading of FFPE RNA quality by RNA Integrity Number (RIN) is not recommended for this application.

Determine the DV200 (percentage of RNA in the sample that is >200 nt) using the analysis mode described in Table 6. RNA molecules must be >200 nt for efficient conversion to cDNA library. Consult Table 7 for DV200-based RNA input recommendations.

Table 7 RNA input guidelines based on DV200 score

DV200 Score	RNA Input Guidelines
DV200 ≥ 20%	50 ng RNA recommended (up to 200 ng may be used)
DV200 < 20%	Not recommended for further processing

4 Place 50 ng of each FFPE RNA sample in 10 µL of nuclease-free water into wells of a thermal cycler-compatible strip tube or PCR plate and hold on ice.

FFPE RNA library preparation steps continue in "Step 2. Add 2X Priming Buffer to all samples and fragment intact RNA samples" below.

Intact RNA samples

The instructions in this section are for intact (non-FFPE) RNA samples. For FFPE-derived RNA samples, see page 17.

1 Prepare intact total RNA from each fresh or fresh-frozen sample. The optimized library preparation protocol uses 50 ng of total RNA in a 10 µL volume of nuclease-free water.

NOTE The SureSelect XT HS2 RNA system supports use of 10–200 ng RNA input. Use of <50 ng RNA for the SureSelect Cancer CGP Assay may reduce yield and target coverage.

2 Use a small-volume spectrophotometer to determine the RNA concentration and the 260/280 and 260/230 absorbance ratio values for the sample. High-quality RNA samples are indicated by values of approximately 1.8 to 2.0 for both ratios.

3 Place 50 ng of each intact RNA sample in 10 µL of nuclease-free water into wells of a thermal cycler-compatible strip tube or PCR plate and hold on ice.

Studies investigating FFPE-derived experimental samples should also include a well characterized, intact control RNA sample, in order to differentiate performance issues related to sample quality from other factors. Agilent's QPCR Human Reference Total RNA (supplied at 1 µg/µL) is recommended for this purpose. Dilute to 5 ng/µL in nuclease-free water before use.

NOTE Intact RNA samples and FFPE RNA samples must be placed in a separate strip tubes or PCR plates, since these sample types are processed under different conditions in the following section. After fragmentation of intact RNA, samples can be reformatted for co-processing on a single plate or strip, beginning with first-strand cDNA synthesis on page 19.

Step 2. Add 2X Priming Buffer to all samples and fragment intact RNA samples

In this step, all RNA samples (both FFPE-derived and intact) are combined with 2X Priming Buffer, containing primers used for cDNA synthesis in addition to fragmentation agents. The intact RNA samples, only, are then chemically-fragmented by incubation at elevated temperature. The FFPE-derived RNA samples are already sufficiently fragmented for library preparation and are held on ice after 2X Priming Buffer addition to prevent further fragmentation.

1 Add 10 µL of 2X Priming Buffer to each RNA sample well, containing 50 ng of either FFPE RNA or intact RNA. Mix well then spin briefly and hold the samples on ice.

2 Transfer the intact RNA samples to a thermal cycler and run the program in Table 8.

Leave the FFPE-derived RNA samples on ice during this step.

Table 8 Thermal cycler program for fragmentation of intact RNA samples (20 µl vol)

Step	Temperature	Time
Step 1	94°C	4 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

3 Once the thermal cycler program reaches the 4°C Hold step, transfer the fragmented RNA samples to ice.

Proceed immediately to "Step 3. Synthesize first-strand cDNA" to continue processing all RNA samples.

Step 3. Synthesize first-strand cDNA

CAUTION The First Strand Master Mix used in this step is viscous. Mix thoroughly by vortexing where directed in the protocol. Pipetting up and down is not sufficient to mix this reagent.

The First Strand Master Mix is provided with actinomycin D already supplied in the mixture. Do not supplement with additional actinomycin D.

1 Preprogram a thermal cycler as shown in Table 9; pause until use in step 5.

Table 9 Thermal cycler program for first-strand cDNA synthesis (28 µl vol)

Step	Temperature	Time
Step 1	25°C	10 minutes
Step 2	37°C	40 minutes
Step 3	4°C	Hold

2 Vortex the thawed vial of First Strand Master Mix for 5 seconds at high speed to ensure homogeneity.

3 Add 8.5 µL of First Strand Master Mix to each RNA sample well.

4 Mix well by pipetting up and down 15–20 times or seal the wells and vortex at high speed for 5–10 seconds. Spin briefly to collect the liquid.

5 Place the samples in the thermal cycler, and resume the program in Table 9.

Step 4. Synthesize second-strand cDNA

CAUTION The Second Strand Enzyme Mix used in this step is viscous. Mix thoroughly by vortexing where directed in the protocol. Pipetting up and down is not sufficient to mix this reagent.

1 Once the thermal cycler program in Table 9 begins the 4°C hold step, transfer the samples to ice.

2 Preprogram a thermal cycler as shown in Table 10; pause until use in step 7.

Table 10 Thermal cycler program for second-strand synthesis (58 µl vol)

Step	Temperature	Time
Step 1	16°C	60 minutes
Step 2	4°C	Hold

3 Vortex the thawed vials of Second Strand Enzyme Mix and of Second Strand Oligo Mix at high speed for 5 seconds to ensure homogeneity.

4 Add 25 µL of Second Strand Enzyme Mix to each sample well. Keep on ice.

5 Add 5 µL of Second Strand Oligo Mix to each sample well, for a total reaction volume of 58.5 µL. Keep on ice.

6 Mix well by pipetting up and down 15–20 times or seal the wells and vortex at high speed for 5–10 seconds. Spin briefly to collect the liquid.

7 Place the plate or strip tubes in the thermal cycler, and resume the program in Table 10.

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

Step 5. Purify cDNA using AMPure XP Beads

Once the thermal cycler program in Table 10 reaches the 4°C hold step, purify the cDNA using room temperature AmpPure XP Beads.

Critical purification protocol parameters are summarized for experienced users in Table 11. A video demonstrating the AmpPure XP Bead purification protocol is available at Agilent.com. (Perform all purification steps in plates or strip tubes as described below; do not transfer samples to 1.5 ml tubes as shown in the video demonstration.)

Table 11 AMPure XP bead cDNA cleanup parameters

Parameter	Value
Volume of RT AMPure XP bead suspension added to each sample well	105 µL
Final elution solvent and volume	52 µL nuclease-free water
Amount of eluted sample transferred to fresh well	Approximately 50 µL

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

NOTE The freshly-prepared 70% ethanol may be used for all purification steps run on the same day. Consult the workflow summary on page 10 to determine how many same-day purification steps will be run.

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

3 Transfer the cDNA samples from the thermal cycler to room temperature, then add 105 µL of the bead suspension to each sample well.

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

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

6 Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 2 to 5 minutes).

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

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

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

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

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

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

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

NOTE Do not dry the bead pellet to the point that the pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

13 Elute the cDNA by adding 52 µL of nuclease-free water to each sample well.

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

15 Incubate for 2 minutes at room temperature.

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

17 Remove 50 µL of cleared supernatant to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

The purified cDNA is ready for NGS library preparation; proceed to "Library Preparation and Pre-capture Amplification" on page 30 to continue processing the cDNA samples. NGS library preparation from fragmented gDNA samples may be performed in parallel; proceed to page 23 for gDNA sample processing instructions.

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

3
DNA-Specific Workflow Steps

This section describes the steps to prepare input gDNA samples and fragment the input DNA either by enzymatic fragmentation or by mechanical shearing to a target fragment length suitable for NGS with 2 x 150 read length. The protocols include conditions for both FFPE-derived gDNA samples (see page 24) and intact DNA from fresh or fresh-frozen samples (see page 25).

If you are preparing RNA libraries (only), use the RNA sample preparation instructions starting on page 16 and skip the instructions in this section.

If you are preparing both DNA and RNA libraries in the same run, see page 16 for DNA & RNA assay workflow synchronization guidelines.

Step 1. Prepare and qualify the genomic DNA samples

FFPE DNA samples

The instructions in this section are for FFPE-derived DNA samples. For intact (non-FFPE) DNA samples, instead follow the instructions on page 25.

Samples are obtained from tissue resection (tissue curls or sections on slide), with use of a minimum of 3 sections of 5 µm each recommended. FFPE tumor samples should have ≥15% tumor content (measured by haemotoxylin & eosin staining).

1 Prepare gDNA from FFPE tissue sections using QIAGEN's QIAamp DNA FFPE Tissue Kit and 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.

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

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

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

Option 1: Qualification using the Agilent Genomic DNA ScreenTape assay DIN score

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

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

b Consult Table 12 for DIN score-based input DNA input guidelines.

Table 12 DNA input guidelines based on DNA Integrity Number (DIN) score

DIN Score	DNA Input Guidelines
DIN > 3	50 ng DNA recommended
DIN 2–3	50 ng DNA required, maximum amount DNA available (up to 200 ng) recommended
DIN <2	Not recommended for further processing

Option 2: Qualification using the Agilent NGS FFPE QC Kit

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

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

b Consult Table 13 for ΔΔCq score-based input DNA input guidelines.

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

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

Table 13 DNA input guidelines based on ΔΔCq DNA integrity score

ΔΔCq Score	DNA Input Guidelines
ΔΔCq≤1*	50 ng DNA recommended, quantified by Qubit Assay
ΔΔCq>1	50–200 ng of amplifiable DNA, based on qPCR quantification

* FFPE samples with ΔΔCq scores ≤1 should be treated like non-FFPE samples for DNA input amount determinations. For samples of this type, make sure to use the DNA concentration determined by the Qubit Assay, instead of the concentration determined by qPCR, to calculate the volume required for 50 ng DNA.

Intact DNA samples

1 Prepare high-quality gDNA from fresh or frozen biological samples 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.

Agilent's OneSeq Human Reference DNA (supplied at 200 ng/μL) is recommended for use as an intact control DNA sample, which can be included in runs in order to differentiate performance issues related to sample quality from other factors. When using this intact control DNA sample, proceed directly to the appropriate DNA fragmentation protocol below for dilution and fragmentation instructions.

Step 2. Fragment the DNA

In this step the appropriate gDNA samples are fragmented either by enzymatic fragmentation or by mechanical shearing

NOTE The SureSelect XT HS2 DNA system supports use of 10–200 ng DNA input. Use of <50 ng DNA for the SureSelect Cancer CGP Assay may reduce yield and target coverage.

Method 1: Enzymatic DNA fragmentation

In this step, gDNA samples are fragmented using Agilent's SureSelect Enzymatic Fragmentation Kit. The conditions provided produce fragments suitable for library construction followed by NGS with 2 x 150 read length.

NOTE For FFPE DNA samples, initial DNA fragment size may impact the post-fragmentation size distribution, resulting in fragment sizes shorter than the target range for the recommended 2 x 150-read NGS in some samples. All samples, including low-integrity FFPE samples, should be incubated at 37°C for 15 minutes to generate fragment ends suitable for library construction.

1 In wells of a thermal cycler-compatible strip tube or PCR plate, dilute 50 ng of each gDNA sample with nuclease-free water to a final volume of 7 µL.

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

3 Preprogram a thermal cycler as shown in Table 14; pause until use in step 7.

Table 14 Thermal cycler program for enzymatic fragmentation (10 µl vol)

Step	Temperature	Time
Step 1	37°C	15 minutes
Step 2	65°C	5 minutes
Step 3	4°C	Hold

4 Prepare the appropriate volume of fragmentation master mix by combining the reagents in Table 15. Mix well then spin briefly and keep on ice.

Table 15 Preparation of fragmentation master mix

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

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

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

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

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

7 Immediately place the samples in the thermal cycler and resume the enzymatic fragmentation program in Table 14.

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 and Pre-capture Amplification" on page 30.

NOTE This is not a stopping point in the workflow, and analysis of the enzymatically-fragmented samples is not required before they are used for library preparation. Proceed directly to page 30.

Method 2: Mechanical DNA shearing with Covaris

In this step, gDNA samples are sheared using conditions optimized for either high-quality or FFPE DNA in a 50-µL shearing volume. The conditions provided produce fragments suitable for NGS with 2 x 150 read length. See Table 16 for shearing parameter guidelines.

Table 16 Covaris shearing parameter summary

Parameter	Value
Target fragment size	180 to 250 bp*
Shearing duration for FFPE DNA samples	240 seconds
Shearing duration for intact DNA samples	2 × 120 seconds

* For FFPE DNA samples, initial DNA fragment size may impact the post-fragmentation size distribution, resulting in fragment sizes shorter than the target size range shown here. All FFPE samples, including low-integrity samples, should be sheared for 240 seconds to generate fragment ends suitable for library construction.

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

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

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

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

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

- **a** Transfer the 50-µL DNA sample into a Covaris microTUBE.
- **b** Spin the microTUBE for 30 seconds to collect the liquid and to remove any bubbles from the bottom of the tube.
- **c** Secure the microTUBE in the tube holder and shear the DNA with the settings in Table 17.

Table 17 Shear settings for Covaris E-series instrument (SonoLab software v7 or later)

Setting	FFPE DNA	High-quality DNA
Duty Factor	10%	10%
Peak Incident Power (PIP)	175	175
Cycles per Burst	200	200
Treatment Time	240 seconds	2 × 120 seconds (shear 120 sec, spin 10 sec, vortex 5 sec, spin 10 sec, then repeat full sequence once more retaining the sample in the microTUBE throughout process)
Bath Temperature	2° to 8° C	2° to 8° C

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

e 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 d.

The 50-µL sheared DNA samples are now ready for NGS sequencing library preparation, beginning with end repair/dA-tailing. Proceed to "Library Preparation and Pre-capture Amplification" on page 30.

NOTE This is not a stopping point in the workflow, and analysis of the sheared samples is not required before they are used for library preparation. Proceed directly to page 30.

4
DNA/RNA Workflow Steps: Library Prep and Hybridization to SureSelect Cancer CGP Assay Probes

The first module in this section describes the steps to prepare NGS libraries from gDNA or cDNA fragments. For each sample to be sequenced, an individual dual-indexed library is prepared. To process multiple samples, the protocol includes steps for preparation of reagent mixtures with overage, which are afterward distributed to the DNA library samples. Mixtures for preparation of 8 or 24 samples are shown in tables as examples.

The second module in this section describes hybridization of the prepared gDNA or cDNA libraries to the appropriate SureSelect Cancer CGP Assay Probe. Target-enriched libraries are then amplified and analyzed prior to pooling for NGS.

Library Preparation and Pre-capture Amplification

This workflow segment uses the components listed in Table 18. Remove the listed reagents from cold storage, and prepare as directed before use (refer to the *Where Used* column).

Table 18 Reagents thawed before use in protocol

Storage Location	Kit Component	Preparative Steps	Where Used
For DNA-input samples get reagents from the *SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR)* box, stored at –20°C For RNA-input samples get reagents from the *SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR)* box, stored at –20°C	Ligation Buffer (purple cap or bottle)	Thaw on ice (may require >20 minutes) then keep on ice, vortex to mix	page 31
	T4 DNA Ligase (blue cap)	Place on ice just before use, invert to mix	page 31
	End Repair-A Tailing Buffer (yellow cap or bottle)	Thaw on ice (may require >20 minutes) then keep on ice, vortex to mix	page 32
	End Repair-A Tailing Enzyme Mix (orange cap)	Place on ice just before use, invert to mix	page 32
	DNA samples: XT HS2 Adaptor Oligo Mix (white cap) — **RNA** samples: XT HS2 RNA Adaptor Oligo Mix (green cap)	Thaw on ice then keep on ice, vortex to mix	page 32
	Herculase II Fusion DNA Polymerase (red cap)	Place on ice just before use, mix by pipetting	page 35
	5× Herculase II Buffer with dNTPs (clear cap)	Thaw on ice then keep on ice, vortex to mix	page 35
–20°C	SureSelect XT HS2 Index Primer Pairs **DNA** samples: Index Pairs 1-16 (blue + white strips) or Index Pairs 1-96 (orange plate) — **RNA** samples: Index Pairs 17-32 (black + red strips) or Index Pairs 97-192 (blue plate)	Thaw on ice then keep on ice, vortex to mix	page 35
+4°C	**DNA** samples: SureSelect DNA AMPure XP Beads — **RNA** samples: SureSelect RNA AMPure XP Beads	Equilibrate at room temperature for at least 30 minutes before use, vortex to mix	page 33 and page 36

Step 1. Prepare the ligation master mix

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

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

1 Prepare the appropriate volume of ligation master mix by combining the reagents in Table 19.

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

Keep at room temperature for 30–45 minutes before use on page 32.

Table 19 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	598 µL
T4 DNA Ligase (blue cap)	2 µL	18 µL	52 µL
Total	25 µL	225 µL	650 µL

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

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

Step 2. Repair and dA-tail the DNA 3' ends

CAUTION The End Repair-A Tailing Buffer used in this step is viscous. Make sure to follow the mixing instructions in step 2 and step 3 on page 32.

1 Preprogram a thermal cycler as shown in Table 20; pause until use in step 5.

Table 20 Thermal cycler program for end repair/dA-tailing (70 µl vol)

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

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

3 Prepare the appropriate volume of dA-tailing master mix by combining the reagents in Table 21.

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

Table 21 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 µL	144 µL	416 µL
End Repair-A Tailing Enzyme Mix (orange cap)	4 µL	36 µL	104 µL
Total	20 µL	180 µL	520 µL

4 Add 20 µL of the end repair/dA-tailing master mix to each sample well containing 50 µL of DNA (either fragmented gDNA or purified cDNA fragments). Mix by pipetting up and down 15–20 times using a pipette set to 50 µL or cap the wells and vortex at high speed for 5–10 seconds.

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

Step 3. Ligate the molecular-barcoded adaptor

1 Once the thermal cycling program in Table 20 reaches the 4°C Hold step, transfer the samples to ice. Preprogram the cycler as show in Table 22; pause until use in step 4.

Table 22 Thermal cycler program for ligation (100 µl vol)

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

2 To each end-repaired/dA-tailed DNA sample (approximately 70 µL), add 25 µL of the ligation master mix that was prepared on page 31 and kept at room temperature. Mix by pipetting up and down at least 10 times using a pipette set to 70 µL or cap the wells and vortex at high speed for 5–10 seconds. Briefly spin the samples.

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

- For **DNA** input libraries–5 µL of XT HS2 Adaptor Oligo Mix (white-capped tube)
- For **RNA** input libraries–5 µL of XT HS2 RNA Adaptor Oligo Mix (green-capped tube)

Mix by pipetting up and down 15–20 times using a pipette set to 70 µL or cap the wells and vortex at high speed for 5–10 seconds.

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

4 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and resume the thermal cycling program in Table 22.

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

Step 4. Purify libraries using AMPure XP Beads

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

Critical purification protocol parameters are summarized for experienced users in Table 23. A video demonstrating the AmpPure XP Bead purification protocol is available at Agilent.com. (Perform all purification steps in plates or strip tubes as described below; do not transfer samples to 1.5 mL tubes as shown in the video demonstration.)

Table 23 AMPure XP bead cleanup parameters after adaptor ligation

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

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

NOTE The freshly-prepared 70% ethanol may be used for all purification steps run on the same day. Consult the workflow summary on page 10 to determine how many same-day purification steps will be run.

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

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

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

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

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

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

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

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

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

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

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

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

NOTE Do not dry the bead pellet to the point that the pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

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

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

15 Incubate for 2 minutes at room temperature.

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

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

Step 5. Amplify the pre-capture libraries

1 Determine the appropriate index pair assignment for each sample. See Table 67 on page 80 through Table 70 on page 83 for nucleotide sequences of the 8 bp index portion of the primers used to amplify the DNA libraries in this step.

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

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

When using index pairs provided in strip tubes in step 5 on page 35, verify the strip tube orientation using the numeral (**1**, **9**, **17** or **25**) etched adjacent to the lowest-numbered index and the strip barcode adjacent to the highest-numbered index. Pierce the foil seal of the appropriate well with a pipette tip just before pipetting the solution.

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

2 Preprogram a thermal cycler as shown in Table 24; pause until use in step 6.

Table 24 Pre-capture PCR thermal cycler program (50 µl vol; heated lid ON)

Segment	Number of Cycles*	Temperature	Time
1	1	98°C	2 minutes
2	FFPE DNA input: 12 cycles Intact DNA input: 9 cycles FFPE RNA input: 15 cycles Intact RNA input: 12 cycles	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

* See *Troubleshooting* on page 86 for PCR cycle number optimization recommendations for low-input libraries and for remediation of low-yield libraries.

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 25, on ice. Mix well on a vortex mixer.

Table 25 Preparation of pre-capture PCR reaction mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
5× Herculase II Buffer with dNTPs (clear cap)	10 µL	90 µL	260 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	9 µL	26 µL
Total	11 µL	99 µL	286 µL

4 Add 11 µL of the PCR reaction mixture prepared in Table 25 to each sample well containing purified DNA library (34 µL).

5 Add 5 µL of the appropriate SureSelect XT HS2 Index Primer Pair to each reaction.

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

6 Before adding the samples to the thermal cycler, resume the thermal cycling program in Table 24 to bring the temperature of the thermal block to 98°C. Once the cycler has reached 98°C, immediately place the sample plate or strip tube in the thermal block and close the lid.

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

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

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

Step 6. Purify amplified libraries using AMPure XP Beads

Once the thermal cycler program in Table 24 reaches the 4°C hold step, purify the libraries using room-temperature AmpPure XP Beads.

Critical purification protocol parameters are summarized for experienced users in Table 26.

Table 26 AMPure XP bead cleanup parameters after pre-capture PCR

Parameter	Value
Volume of RT AMPure XP bead suspension added to each sample well	50 µL
Final elution solvent and volume	15 µL nuclease-free water
Amount of eluted sample transferred to fresh well	Approximately 14 µL

1. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 8.
2. Mix the room-temperature AMPure XP Beads well until homogeneous and consistent in color.
3. Transfer the library DNA samples from the thermal cycler to room temperature, then add 50 µL of the bead suspension to each sample well.
4. Mix by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds then spin briefly to collect the samples, being careful not to pellet the beads.
5. Incubate the bead suspensions for 5 minutes at room temperature.
6. Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 minutes).
7. Keep the plate or strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
8. Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 µL of fresh 70% ethanol in each sample well.
9. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
10. Repeat step 8 and step 9 once for a total of two washes.
11. Cap the wells, then briefly spin the samples to collect the residual ethanol. Return the samples to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
12. Dry the samples by placing the unsealed plate or strip tube on the thermal cycler, set to hold samples at 37°C, until the residual ethanol has just evaporated (up to 2 minutes).

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

NOTE Do not dry the bead pellet to the point that the pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

13. Elute the library DNA by adding 15 µL of nuclease-free water to each sample well.
14. Mix by pipetting up and down 10–15 times or cap the wells and vortex at high speed for 5 seconds. Verify that all beads have been resuspended, with no visible clumps in the suspension or bead pellets retained on the sides of the wells. If samples were vortexed, spin briefly to collect the liquid, being careful not to pellet the beads.
15. Incubate for 2 minutes at room temperature.

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

17 Remove the cleared supernatant (approximately 14 µL) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

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

Step 7. QC and quantify the pre-capture libraries

Analyze a sample of each library using one of the platforms listed in Table 27. Follow the instructions in the linked user guide provided for each assay.

Table 27 Pre-capture library analysis options

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

Each analysis method provides an electropherogram showing the size distribution of fragments in the sample and tools for determining the concentration of DNA in the sample. See Table 28 for fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided in Figure 2 and Figure 3 to illustrate typical results.

Table 28 Pre-capture library qualification guidelines

Input type	Expected library DNA fragment size peak position
FFPE DNA	200 to 400 bp
Intact DNA	270 to 400 bp
RNA (FFPE or Intact)	200 to 700 bp

Observation of a low molecular weight peak, in addition to the expected library fragment peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to that seen in example electropherogram in Figure 3. See *Troubleshooting* on page 87 for additional considerations.

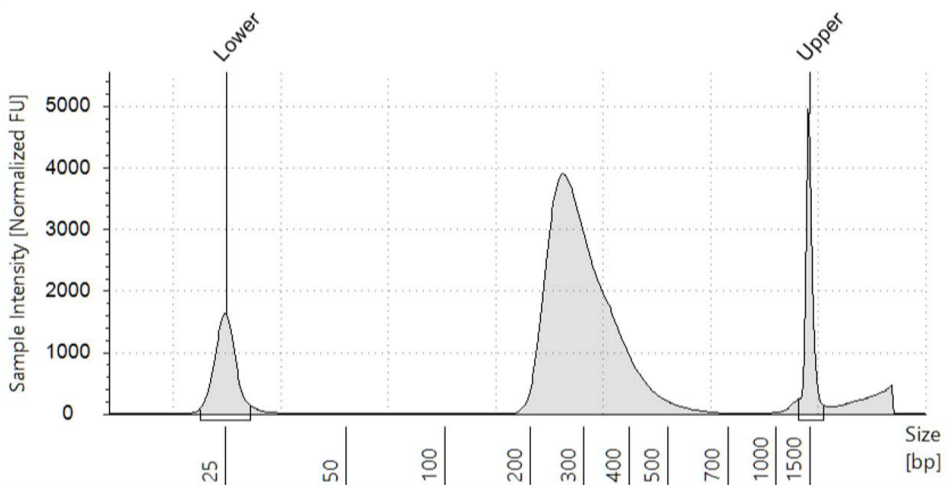

Figure 2 Pre-capture library prepared from an enzymatically fragmented FFPE gDNA sample, analyzed using a D1000 ScreenTape assay.

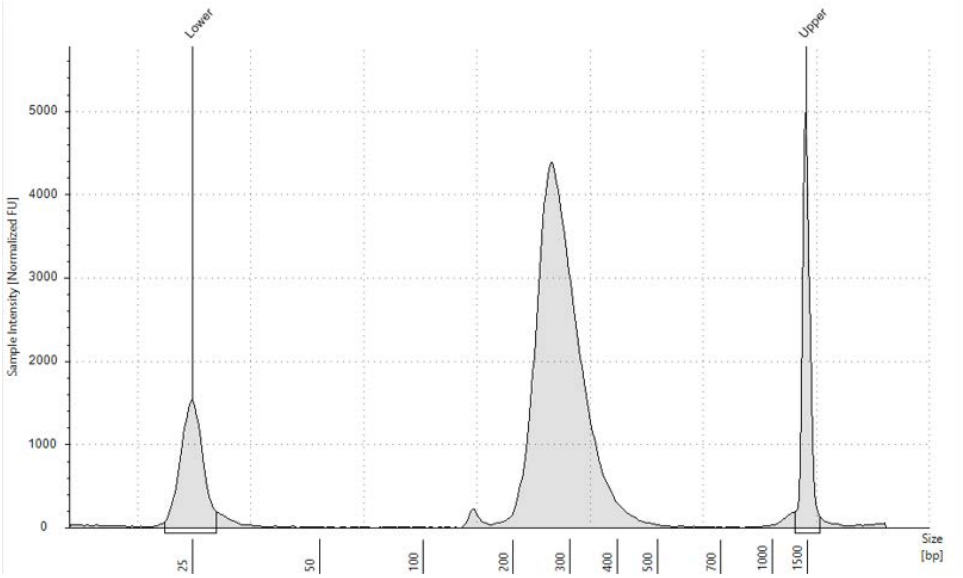

Figure 3 Pre-capture library prepared from an FFPE RNA sample, analyzed using a D1000 ScreenTape assay.

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

Hybridization, Capture and Post-capture Amplification

In this workflow segment, the prepared gDNA libraries are hybridized to the SureSelect Cancer CGP Assay Probe(s). For each sample library prepared, do one hybridization and capture. The captured libraries are pooled for multiplexed sequencing after all capture steps are complete.

The hybridization reaction requires 500-1000 ng of prepared library for the DNA assay and 200 ng of prepared library for the RNA assay, in a volume of 12 µL.

This workflow segment uses the components listed in Table 29. Remove the listed reagents from cold storage, when required, and prepare as directed before use (refer to the *Where Used* column).

Table 29 Reagents for Hybridization and Capture

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), stored at –20°C	SureSelect XT HS2 Blocker Mix (blue cap)	Thaw and keep on ice, vortex to mix	page 40
	SureSelect RNase Block (purple cap)	Thaw and keep on ice, vortex to mix	page 40
	SureSelect Fast Hybridization Buffer (bottle)	Thaw and keep at room temperature	page 41
–80°C	For **DNA-input** libraries: SureSelect Cancer CGP Assay Probe DNA (red cap) — For **RNA-**input libraries: SureSelect Cancer CGP Assay Probe RNA (blue cap)	Thaw and keep on ice, vortex to mix	page 41
+4°C	SureSelect Streptavidin Beads (clear cap or bottle)	Remove from 4°C just before use, vortex to mix	page 42
SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), stored at RT	SureSelect Binding Buffer (bottle)	Ready to use	page 42
	SureSelect Wash Buffer 1 (bottle)	Ready to use	page 42
	SureSelect Wash Buffer 2 (bottle)	Ready to use	page 42
SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), stored at –20°C	Herculase II Fusion DNA Polymerase (red cap)	Place on ice just before use, pipette to mix	page 44
	5× Herculase II Buffer with dNTPs (clear cap)	Thaw and keep on ice, vortex to mix	page 44
	SureSelect Post-Capture Primer Mix (clear cap)	Thaw and keep on ice, vortex to mix	page 44
+4°C	For **DNA-**input libraries: SureSelect DNA AMPure XP Beads (bottle) — For **RNA-**input libraries: SureSelect RNA AMPure XP Beads (bottle)	Equilibrate at room temperature for at least 30 minutes before use, vortex to mix	page 44

Step 1. Hybridize libraries to the SureSelect Cancer CGP Assay Probe

1 Preprogram a thermal cycler as shown in Table 30; pause until samples are loaded in step 4.

Table 30 Pre-programmed thermal cycler program for hybridization (30 µl vol; heated lid ON)

Segment Number	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	65°C	10 minutes
3	1	65°C	1 minute (Pause cycler here for reagent addition, see step 7 on page 41)
4	60	65°C	1 minute
		37°C	3 seconds
5	1	65°C	Hold briefly until ready to begin capture steps on page 42

2 Place 1000 ng of each prepared gDNA library or 200 ng of each cDNA library (prepared from RNA samples) into the hybridization plate or strip tube wells. Bring the final volume in each well to 12 µL using nuclease-free water.

If 1000 ng gDNA library is not available for any of the DNA assay samples, use the maximum amount of library 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 in Table 30**,** 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, as described in step 7 on page 41.

During Segments 1 and 2 of the thermal cycling program, begin preparing the additional hybridization reagents as described in step 5 below and step 6 on page 41. 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 31. Prepare the amount required for the number of hybridization reactions in the run, plus excess. Mix well and keep on ice.

Table 31 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. Do not keep solutions containing the probe at room temperature for extended periods.

6 Prepare the probe hybridization mix according to Table 32. **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 32 Preparation of probe hybridization mix

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
SureSelect Cancer CGP Assay Probe (DNA probe **OR** RNA probe)*	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

* Add either SureSelect Cancer CGP Assay Probe DNA OR SureSelect Cancer CGP Assay Probe RNA; **do not combine DNA and RNA assay probes in the same hybridization reaction**.

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 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 library 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 beads for capture

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

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

Step 3. Capture the hybridized libraries

1. After all streptavidin bead preparation steps are complete, and once the hybridization thermal cycling program reaches the 65°C hold step (Segment 5; see Table 30 on page 40), transfer the samples to room temperature.
2. Immediately transfer the entire volume (approximately 30 µL) of each hybridization mixture to wells containing 200 µL of washed streptavidin beads using a multichannel pipette.

   Pipette up and down 5–8 times to mix then seal the wells with fresh caps.
3. Incubate the capture plate or strip tube on a 96-well plate mixer, mixing vigorously (at 1400–1900 rpm), for 30 minutes at room temperature.

   Make sure the samples are properly mixing in the wells.
4. During the 30-minute incubation for capture, prewarm SureSelect Wash Buffer 2 at 70°C as described below.
   a. Place 200-µL aliquots of Wash Buffer 2 in wells of a fresh 96-well plate or strip tubes. Aliquot 6 wells of buffer for each DNA sample in the run.
   b. Cap the wells and then incubate in the thermal cycler held at 70°C until used in step 9.
5. When the 30-minute capture incubation period initiated in step 3 is complete, spin the samples briefly to collect the liquid.
6. Put the plate or strip tube in a magnetic separator to collect the beads. Wait until the solution is clear (approximately 1 to 2 minutes), then remove and discard the supernatant.
7. Resuspend the beads in 200 µL of SureSelect Wash Buffer 1. Mix by pipetting up and down 15–20 times, until beads are fully resuspended.
8. Put the plate or strip tube in the magnetic separator. Wait for the solution to clear (approximately 1 minute), then remove and discard the supernatant.

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

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

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

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

- **a** Resuspend the beads in 200 µL of 70°C prewarmed Wash Buffer 2. Pipette up and down 15–20 times, until beads are fully resuspended.
- **b** Seal the wells with fresh caps and then vortex at high speed for 8 seconds. Spin the plate or strip tube briefly to collect the liquid without pelleting the beads.

  Make sure the beads are in suspension before proceeding.
- **c** Incubate the samples 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.

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

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

Step 4. Amplify the captured libraries

1 Preprogram a thermal cycler as shown in Table 33; pause until use in step 5.

Table 33 Post-Capture PCR thermal cycler program (50 µl vol; heated lid ON)

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	13	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

2 Prepare the appropriate volume of post-capture PCR reaction mix, as described in Table 34, on ice. Mix well on a vortex mixer.

Table 34 Preparation of post-capture PCR reaction mix

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

3 Add 25 µL of the PCR reaction mix prepared in Table 34 to each sample well containing 25 µL of bead-bound target-enriched DNA.

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

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

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

   The streptavidin beads can be discarded at this time.

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

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

Step 5. Purify the final libraries using AMPure XP Beads

Purify the amplified libraries using room-temperature AmpPure XP Beads. Critical purification protocol parameters are summarized for experienced users in Table 35.

Table 35 AMPure XP bead cleanup parameters after post-capture PCR

Parameter	Value
Volume of RT AMPure XP bead suspension added to each sample well	50 µL
Final elution solvent and volume	25 µL Low TE Buffer
Amount of eluted sample transferred to fresh well	Approximately 24 µL

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

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

3 Add 50 µL of the bead suspension to each amplified DNA sample (approximately 50 µL) in the PCR plate or strip tube well.

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

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

6 Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 2 to 5 minutes).

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

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

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

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

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

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

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

NOTE Do not dry the bead pellet to the point that the pellet appears cracked. Elution efficiency is significantly decreased when the bead pellet is excessively dried.

13 Elute the library DNA by adding 25 µL of Low TE buffer to each sample well.

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

15 Incubate for 2 minutes at room temperature.

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

17 Remove the cleared supernatant (approximately 24 µL) to a fresh PCR plate or strip tube sample well and keep on ice. You can discard the beads at this time.

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

Step 6. QC and quantify final libraries

Analyze a sample of each library using one of the platforms listed in Table 36. Follow the instructions in the linked user guide provided for each assay.

Table 36 Post-capture library analysis options

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

Each analysis method provides an electropherogram showing the size distribution of fragments in the sample and tools for determining the concentration of DNA in the sample. See Table 37 for fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided in Figure 4 and Figure 5 to illustrate typical results.

Table 37 Post-capture library qualification guidelines

Input type	Expected library DNA fragment size peak position
DNA (FFPE or intact)	200 to 450 bp
RNA (FFPE or Intact)	200 to 700 bp

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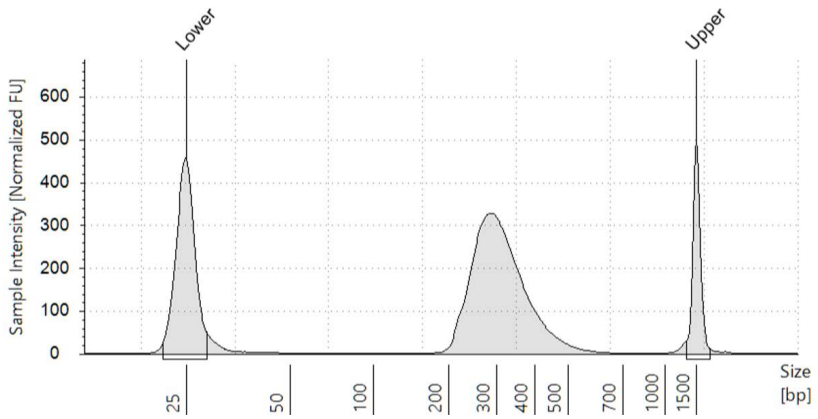

Figure 4 Post-capture library prepared from an enzymatically fragmented FFPE gDNA sample, analyzed using a High Sensitivity D1000 ScreenTape assay.

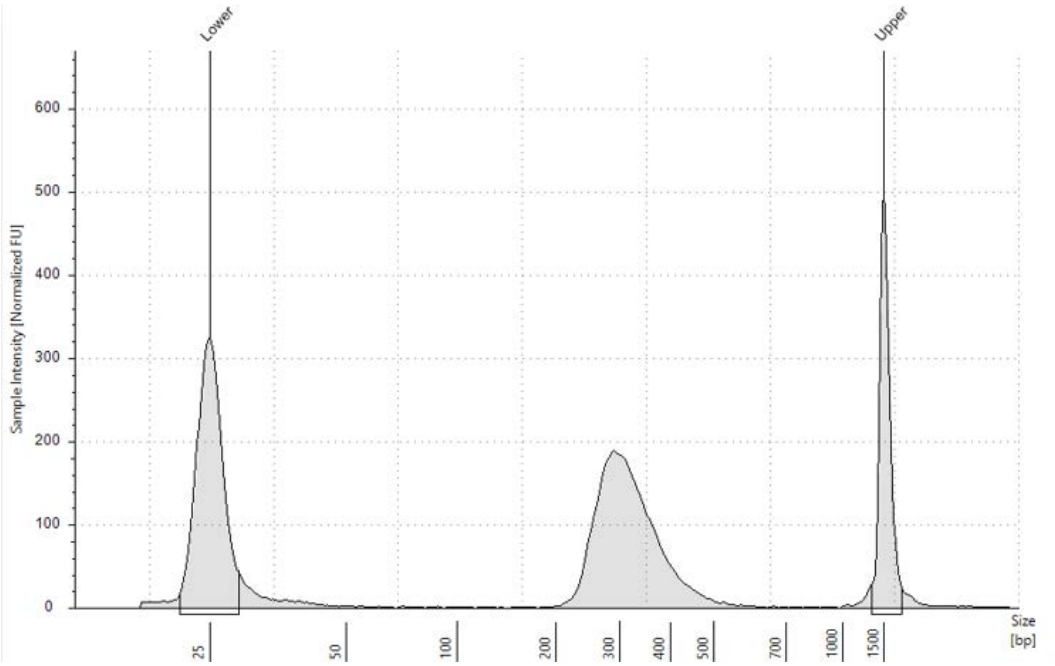

Figure 5 Post-capture library prepared from an FFPE RNA sample, analyzed using a High Sensitivity D1000 ScreenTape assay.

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

5
NGS and Analysis Workflow Steps

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

The SureSelect Cancer CGP libraries are sequenced using standard Illumina paired-end primers and chemistry. The sequencing parameters below are recommended for optimal SureSelect Cancer CGP Assay analysis performance:

- Depth of ≥40M reads per sample for SureSelect Cancer CGP DNA Assay samples
- Depth of ≥10M reads per sample for SureSelect Cancer CGP RNA Assay samples
- Read length of 2 × 150 bp (recommended for optimal translocation detection in DNA samples)

After reads are demultiplexed, Agilent's Alissa Reporter software provides a complete FASTQ-to-Report solution for the SureSelect Cancer CGP assays, processing NGS data from FASTQ format to VCF format, and reporting SNV, InDel, CNV, translocation, RNA fusion and RNA exon skipping calls along with TMB and MSI values. Alternatively, the demultiplexed reads can be pre-processed using Agilent's Genomics NextGen Toolkit (AGeNT) and the processed reads analyzed using the appropriate variant analysis tools.

Step 1. Pool samples for multiplexed sequencing

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the sequencer used, together with the amount of sequencing data required per sample (≥40M reads for each DNA sample and ≥10M reads for RNA samples). If you wish to sequence DNA and RNA libraries together in the same lane, first make separate pools for RNA and DNA samples at the same concentration (e.g., 10 nM in each pool), then combine the RNA and DNA pools at 4 parts DNA pool to 1 part RNA pool.

Combine the libraries such that each indexed library is present in equimolar amounts in the pool using one of the following methods. Use the diluent specified by your sequencing provider, such as Low TE, for the dilution steps.

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

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

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

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

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

is the number of indexes, and

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

Table 38 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM DNA.

Table 38 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 are sequencing DNA and RNA libraries together in the same lane, first make separate pools for RNA and DNA samples at the same concentration (e.g., 10 nM) using either of the methods described above, then combine the RNA and DNA pools at 4 parts DNA pool to 1 part RNA pool.

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

Step 2. Prepare the sequencing samples

The final SureSelect Cancer CGP library pool is ready for sequencing using standard Illumina paired-end primers and chemistry. Each fragment in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform, as shown in Figure 6.

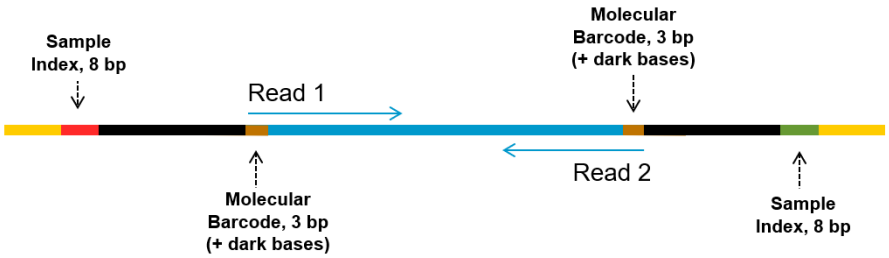

Figure 6 Content of SureSelect Cancer CGP sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), unique dual sample indexes (red and green), duplex molecular barcodes (brown) and the library PCR primers (yellow).

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit and sequence the libraries using an Illumina instrument. Table 39 provides guidelines for use of several instrument and chemistry combinations suitable for this application, including kit configurations compatible with the recommended 2 × 150 bp read length and seeding concentration recommendations. For other Illumina NGS platforms, consult Illumina's documentation for kit configuration and seeding concentration guidelines.

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

Table 39 Illumina kit configuration selection guidelines

Platform	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
NextSeq 500/550	All Runs	2 × 150 bp	300 Cycle Kit	v2.5	1.2–1.5 pM
NextSeq 2000	All Runs	2 × 150 bp	300 Cycle Kit	v1, v2, or v3	1000 pM
HiSeq 4000	All Runs	2 × 150 bp	300 Cycle Kit	v1	300–400 pM
NovaSeq 6000	Standard Workflow Runs	2 × 150 bp	300 Cycle Kit	v1.0 or v1.5	300–600 pM
NovaSeq 6000	Xp Workflow Runs	2 × 150 bp	300 Cycle Kit	v1.0 or v1.5	200–400 pM

Step 3. Sequence the libraries

Set up the sequencing run to generate Read 1 and Read 2 FASTQ files for each sample using the instrument's software in standalone mode or using an Illumina run management tool such as Local Run Manager (LRM), Illumina Experiment Manager (IEM) or BaseSpace. Enter the appropriate **Cycles** or **Read Length** value for your library read length and using 8-bp dual index reads. See Table 40 showing example settings for 2x150 bp sequencing.

Table 40 Run settings for 2x150 bp sequencing

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

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

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

- Each of the sample-level indexes (i7 and i5) requires an 8-bp index read. For complete index sequence information, see page 79.
- No custom primers are used for SureSelect XT HS2 library sequencing. Leave all *Custom Primers* options for *Read 1*, *Read 2*, *Index 1* and *Index 2* primers cleared/deselected during run setup.
- Turn off any adaptor trimming tools included in Illumina's run setup and read processing software applications. Adaptors are trimmed in later processing steps using Agilent software tools to ensure proper processing of the adaptors, including the degenerate molecular barcodes (MBCs) in the adaptor sequences.
- For runs set up using Illumina's LRM, IEM, or BaseSpace applications, refer to Illumina's instructions and support resources for setting up runs with custom library prep kits and index kits in the selected software. For use in these applications, the SureSelect XT HS2 index sequences provided in Table 67 through Table 70 should be converted to .tsv/.csv file format or copied to a Sample Sheet according to Illumina's specifications for each application. If you need assistance with SureSelect XT HS2 run setup in your selected application (e.g., generating index files or Sample Sheet templates), contact the SureSelect support team (see page 2) or your local representative.
- If you will use Agilent's Alissa Reporter for downstream analysis, sequence filename requirements and other sequencing setup information is available in the Alissa Reporter software Help, accessed from the *Upload run data* screen by clicking the Help icon (**?**) in the top right corner. If supported by your sequencer, samples may be split across different flow cell lanes when required. Alissa Reporter supports automatic merging of FASTQ files for samples sequenced in multiple lanes.

Step 4. Process the reads to analysis-ready files

Guidelines for sequencing data processing options are outlined below.

1 Generate demultiplexed FASTQ files for each sample using Illumina's bcl2fastq, BCL Convert or DRAGEN software. This process generates paired-end reads based on the dual indexes and removes sequences with incorrectly paired P5 and P7 indexes. Do not use the MBC/UMI trimming options offered in Illumina's demultiplexing software.

   If you are using Agilent's Alissa Reporter software for variant discovery, no further read processing is required prior to FASTQ file uploads. Proceed to page 53**.** If using other analysis software tools, proceed to step 2 below.

2 Complete the FASTQ to BAM pre-processing steps below before analyzing the reads using non-Agilent analysis software.

   The steps below use Agilent's Genomics NextGen Toolkit (AGeNT), a set of Java-based software modules for 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 and review the AGeNT Best Practices document for processing steps suitable for XT HS2 libraries.

   a Remove sequencing adaptors from the reads and extract the MBC sequences using the AGeNT Trimmer module.

      Library fragments include a degenerate molecular barcode (MBC) in each strand (see Figure 6 on page 50). The MBC sequence and dark bases are located at the 5' end of both Read 1 and Read 2. Adaptor trimming should be performed using AGeNT Trimmer module. Other adaptor trimmers may fail to remove the MBC sequences from the opposite adaptor, which can affect alignment quality.

NOTE

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

If demultiplexing using bcl2fastq, MBCs may be masked by including the base mask **N5Y*,I8,I8,N5Y*** (where * is replaced with the remaining read length after subtracting the 5 masked bases, e.g., use **N5Y146,I8,I8,N5Y146** for 2x150 NGS set up as shown in Table 40 on page 51). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

If demultiplexing using BCL Convert, MBCs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y*;I8;I8;N5Y*** (where * is replaced with read length after trimming, e.g., use **N5Y146;I8;I8;N5Y146** for 2x150 NGS set up as shown in Table 40 on page 51). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

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

b For DNA assay libraries: Align the trimmed reads and add MBC tags to the aligned BAM files using a suitable tool such as BWA- MEM. Then use the AGeNT CReaK (Consensus Read Kit) tool to generate consensus reads and mark or remove duplicates.

For RNA assay libraries: Aligned the trimmed reads using a suitable RNA data alignment tool. Once alignment is complete, the AGeNT CReaK (Consensus Read Kit) tool can be used in the single- strand consensus mode to generate consensus reads and mark or remove duplicates.

The resulting BAM files are ready for downstream analysis including gene expression and variant discovery. Additional resources for analysis pipeline steps are provided below.

Obtaining BED files: Browser extensible data (BED) files detailing the annotated coordinates of genomic regions included in the SureSelect Cancer CGP probes are available at Agilent's SureDesign site. A targets.txt file listing the genes targeted is also available for each probe.

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

Step 5. Analyze using Alissa Reporter software

NOTE Alternative NGS analysis software tools can also be used for variant discovery. Complete the FASTQ file pre-processing steps on page 52 before analysis using any non-Agilent software tools. Consult the software documentation for file upload parameters and analysis settings appropriate for your research goals.

Agilent's Alissa Reporter analysis software is designed to perform read trimming, alignment of reads to the reference genome, and variant calling for SureSelect Cancer CGP Assay sequencing data, displaying pre-set thresholds in the QC dashboard which are fine-tuned to the SureSelect Cancer CGP assay. This section provides guidelines on how to upload the sample files and set up analysis. See the appropriate Alissa Reporter software Help topic for more detailed information.

NOTE Uploading and analysis of sequencing files in Alissa Reporter can be fully automated using Amazon Web Services (AWS) S3. See the Alissa Reporter Amazon Web Services Technical Guide for more information.

1 Upload the set of FASTQ files (format .fastq or .fastq.gz) for the run to the Alissa Reporter analysis software. From the software Home page, click **Upload a new run**. Forward and reverse reads from the same sample must be uploaded in the same run. Cancer CGP DNA and RNA Assay samples may be uploaded in the same run.

   The file upload wizard provides guidance on run and sample attributes entered during file uploads. Values appropriate for required sample attribute fields are summarized in Table 41.

NOTE

The run setup process can be automated using a meta information manifest file (*attribute.yaml)* that is uploaded with the FASTQ files and is used to pre-populate the settings in the upload wizard. After adding the *attribute.yaml* file to the run, click **Parse attribute file** to parse the file data and confirm compatibility. For more information on automated setup including link to an *attribute.yaml* file template, click the Help icon (**?**) on the *Upload run data* page.

Sample attribute guidelines: Each Cancer CGP DNA Assay tumor sample sequence file to be analyzed must be designated as a ***Target sample*** type during file upload. Each reference DNA sample sequence file must be designated as either a ***Matched reference sample*** or an ***Unmatched reference sample*** during file upload. SureSelect Cancer CGP RNA Assay tumor samples do not need to be designated as target samples or associated with reference samples for analysis.

Table 41 Alissa Reporter sample file upload settings for the SureSelect Cancer CGP Assays

Field or menu	Affected Samples	Value
Application	All samples	***Cancer CGP DNA*** OR ***Cancer CGP RNA***
Application chemistry	All samples	***XTHS2***
Sample type	Cancer CGP DNA samples only	Select ***Target sample*** for the experimental tumor sample DNA. Select ***Matched reference sample**** for a normal reference DNA sample from same individual OR ***Unmatched reference sample***† for a normal reference DNA sample from another source (e.g., Agilent's OneSeq Human Reference DNA)
Reference sample	Cancer CGP DNA samples designated as *Target sample* only	For each ***Target sample*** in the run, expand the **Reference sample** menu and select the appropriate reference sample name. ***Matched reference sample*** files must be uploaded in the same run as the corresponding ***Target sample*** files. ***Unmatched reference sample*** files can be uploaded in the same run or a reserved unmatched reference can be assigned from a previously uploaded run. If the *Target sample* will be analyzed without a reference sample, select ***No reference sample*** from the menu. Use of a reference sample is required for CNV calling.
In silico filter	Cancer CGP DNA *Target samples* and Cancer CGP RNA samples (not entered for DNA *Reference samples*)	To display analysis results for the full Cancer CGP Probe design, without filtration for specific genes or regions, retain the default setting of ***No filter***. Customized in silico filters may be created to restrict displayed analysis results to a selected panel of genes and/or genomic regions. For more information on how to set up and use customized filters, click the Help icon (**?**) on the in silico filter selection screen.
Sex	All samples	Select the sex (X and Y chromosome composition) for each sample. It is important to use *Target samples* and *Reference samples* of the same sex to allow CNV calling and tumor-normal SNV/Indel calling on the sex chromosomes.

* Each *Matched reference sample* must be associated with the corresponding *Target sample* in the same Alissa Reporter run upload.

† Each *Unmatched reference sample* must be associated with at least one *Target sample* in the same run upload. Once uploaded the *Unmatched reference sample* may also be associated with additional *Target samples* in later-uploaded runs. To make an unmatched reference sample available for use in later-uploaded runs, the sample must be reserved by setting the **Reserved status** of the sample to *Yes* after uploading.

2 Select the appropriate analysis options for the run. Guidelines for key analysis settings are provided in Table 42 for the Cancer CGP DNA Assay and in Table 43 for the Cancer CGP RNA Assay. Information on each analysis setting can be obtained during run setup or analysis setup by clicking the Help icon (**?**) next to each setting field.

NOTE

Analysis option settings may also be entered or changed after the sequencing files for the run are uploaded.

Table 42 Key Alissa Reporter Analysis Settings for the SureSelect Cancer CGP DNA Assay

Field or menu	Value(s)	Usage guidelines
SNV/Indel Analysis mode	*Tumor-normal*	Use to perform SNV and indel calling with a matched or unmatched reference sample. If no matched or unmatched reference sample is available for the analysis, then the Analysis mode is set to *Tumor-only* and cannot be changed.
	Tumor-only	Use to perform SNV and indel calling without a reference sample.
SNV/Indel Deduplication mode	*MBC* (requires selection of *Hybrid, Single,* or *Duplex* consensus mode)	Select *MBC* deduplication mode, then select *Hybrid* consensus mode for the deduplication settings recommended for high-sensitivity Cancer CGP DNA Assay SNV/Indel analysis. Using these settings, reads that share the same MBC, genomic position, library ID, and orientation are identified as duplicates, with consensus read determination methods optimal for this assay at the recommended sequencing depth. For any consensus mode selection, retaining the default *Minimum number of read pairs per MBC* value(s) is recommended.
	Positional	Deduplication method based on read genomic position, library ID, and orientation without consideration of MBC sequence. If preferred, this mode can be used for Cancer CGP DNA Assay analysis.
SNV/Indel analysis (multiple settings)	Retain defaults for recommended values	The *Minimum variant allele frequency* setting (with default value 0.05) may be reduced to include variants at lower abundance in the sample or increased to exclude lower abundance variants. The associated *Minimum reads supporting variant allele* setting may require co-adjustment. Somatic SNV/indel calling is only done at genomic positions with coverage of at least 6 reads and for variants having an alternative allele frequency of at least 0.001.
CNV analysis (multiple settings)	Retain defaults for recommended values	Settings may be adjusted to include or exclude variants detected at different quality scores or lengths. Alissa Reporter only considers a variant to be a putative CNV if it is at least 500 bp in length.
Translocation analysis (multiple settings)	Retain defaults for recommended values	The *Minimum variant allele frequency* setting (with default value 0.01) may be reduced to include variants at lower abundance in the sample or increased to exclude lower abundance variants. The associated *Minimum reads supporting variant allele* setting may require co-adjustment. Only translocation variants having an alternative allele frequency of at least 0.001 and read coverage of at least 1 are reported.
TMB analysis	*Yes* (default) or *No*	Indicates if calculation of TMB (tumor mutational burden) is to be included in the sample analysis. TMB analysis is not compatible with the use of an in silico filter.
MSI analysis	*Yes* (default) or *No*	Indicates if calculation of MSI (microsatellite instability) is to be included in the sample analysis.

Table 43 Alissa Reporter Analysis Settings for the SureSelect Cancer CGP RNA Assay

Field or menu	Value(s)	Usage guidelines
RNA fusion analysis Deduplication mode	*Positional*	Use of positional deduplication is generally recommended for Cancer CGP RNA samples.
	None	This option may be helpful for identifying low frequency variants in RNA samples because more reads are retained for the variant calling analysis.
RNA fusion analysis	Retain default	*Minimum total supporting reads* setting of 2 is optimal for most RNA fusion events. The total supporting reads is the sum of all junction reads and reads from fusion-spanning read pairs.
Exon skipping analysis	Retain default	Alissa Reporter uses a Transcripts Per Kilobase Million (TPM) normalization method for RNA abundance reporting, with separate TPM values reported for the variant transcript (with exon skipping) and the normal transcript (without exon skipping) in the sample. The *minimum TPM ratio variant/variant+normal* setting controls the relative abundance of an exon skipping variant in the sample required for a positive call. The default threshold value is 0.5. Agilent recommends using known positive and negative samples for MET exon 14-skipping and EGFRvIII to verify and optimize this threshold setting. Calls passing the threshold should be further evaluated for variant TPM value. A minimum variant TPM of 10 is recommended to indicate sufficient expression of the variant transcript for calling.

3 Once all run and sample attributes (required) and analysis settings (optional) are entered, click the **Upload files** button.

To begin analysis immediately after the FASTQ files are uploaded, select **Start analysis when upload is completed** checkbox. When selected, all analysis settings must be entered before file upload begins.

4 If analysis was not initiated during file upload, analyze the run or individual samples after file upload is complete. To analyze the full run, open the run from the *List of Runs* page, then click **Analyze run.** To analyze an individual sample, open the sample from the *List of Samples* page, then click **Analyze sample.** See the considerations for analysis settings in Table 42 (for DNA assay) or Table 43 (for RNA assay) above.

5 Once analysis is complete, the Alissa Reporter software provides a variety of options for viewing and reporting of sample data and run data including the options below:

- To view summary information for a sample or for a run click the icon for the sample or run of interest then open the *Overview* tab.
- To generate a report in .pdf format that includes the full set of run and/or sample attributes, QC metrics and a summary of analysis results, open the run page or the sample page then from the *Overview* tab click the **Create report** button.
- To view the QC metrics results for the run or a specific sample, click the icon for the sample or run of interest, then open the *QC dashboard* tab.
- To view the analysis results for a sample, click the icon for the sample of interest, then open the tab for the relevant type of analysis (*SNV/Indel, CNV, Translocation, TMB* or *MSI* tab for DNA analysis and *RNA fusion* or *Exon skipping* tab for RNA analysis). To view detailed information on an individual variant, click the variant on the list then click one of the available options for viewing the variant details, such as **Open in view** or **Show pileup**. The available results display options vary for different genomic/transcriptomic features.

- To create downloadable output files for the run or a sample, click the **Download file** button from a run page or a sample page *Overview* tab. The available output file types include a QC metrics text file and a variety of application-specific output files such as the *Inaccessible Regions* BED file and variant results summaries in tabular format or Variant Call Format (VCF).

NOTE

Review the retention policies for uploaded sequencer files, data files and analysis results files by clicking the Help icon (**?**) then clicking **Go to help overview** in the Help dialog footer and browsing to the *Data retention policy* topic.

Analysis Considerations

Guidelines for key SureSelect Cancer CGP assay analysis considerations are provided below, with details for analysis using Agilent's Alissa Reporter software included where appropriate. Consult the documentation for your selected analysis software for complete information on analysis algorithm-based requirements, thresholds, precautions and limitations. For Agilent's Alissa Reporter, a full list of variant calling precautions and limitations is available for each assay in the pdf-formatted Analysis Report generated from the dashboard for each analyzed sample or run.

- **SNV/Indel variant allele frequency:** Limitations to detection of SNVs and Indels depend on the coverage and sequencing depth. The Alissa Reporter Cancer CGP Assay uses a default SNV/Indel minimum variant allele frequency (VAF) setting of 0.05. It is possible to adjust this setting to 0.001. However, detection of SNV and Indel variants present at <5% frequency may require analysis using more than 40M reads and lowering this setting could increase the number of false-positive variant calls. Given the expected range of VAF values and the threshold for variant filtering based on VAF, some true-positive variant calls may be discarded due to low VAF.
- **Sex chromosome SNV/Indel variants:** For SNV/indel calling on the sex chromosomes in tumor-normal mode, the sex of the samples is used in the analysis. It is important to use target and reference samples that have the same sex.
- **Reference DNA processing**: When using Agilent's Alissa Reporter, CNV analysis and SNV/Indel analysis in tumor-normal mode both require sequence data from a reference sample (either matched or unmatched). It is recommended to include a matched (non-tumorous tissue) reference for tumor-normal paired analysis. For both matched reference and unmatched reference, it is recommended to process and sequence the reference sample in the same run as the target samples. Alissa Reporter software allows analysis using pre-established unmatched reference sample data from a prior run. However, the potential bias due to batch differences may increase the copy number noise and negatively impact the accuracy of calling. Agilent's OneSeq Human Reference DNA or DNA from any sex-matched control sample that does not contain aberrations can be used as an unmatched reference sample for the Cancer CGP DNA application.
- **CNV analysis:** CNV calls should be reviewed and verified with consideration of adherence to the reference sample requirements and other variant calling factors.

  To enable CNV analysis, sequence data from a reference sample (either matched or unmatched) without copy number aberrations in the regions of interest is required. In order for a reference sample to be considered matched, it must be collected from the same source as the target sample, but from an area consisting of normal/non-tumorous tissue. Unmatched reference samples may also be used. For Agilent's Alissa Reporter software, using a matched reference sample improves the sensitivity of the CNV calling algorithm. Co-processing of reference samples and experimental target samples is recommended.

CNV calling on the sex chromosomes depends on the sex of the sample in the analysis. It is important to provide accurate sex information for each sample.

Consult the selected analysis software documentation for any additional algorithm-based CNV calling reference sample requirements, precautions and limitations.

- **Targeted translocations:** The SureSelect Cancer CGP DNA assay probe design targets selected regions of oncogenic driver genes at specific reported translocation break-ends. A translocation with neither mate read in the targeted regions will not be detected regardless of the abundance of the translocation in the sample.
- **Translocations and repetitive sequences:** Translocations frequently occur in intronic and intergenic regions that are more likely to contain repetitive sequences with lower capture specificity than exonic regions. Due to the low complexity of these regions the sequencing reads are difficult to align accurately to the reference genome. Misaligned reads can result in spurious translocation event calls. Agilent's Alissa Reporter software applies filters to minimize these events.
- **TMB analysis:** The exonic genome coverage size (sum of exonic region sequence) of the SureSelect Cancer CGP Assay Probe DNA is 1.605 Mb. Using Agilent's Alissa Reporter SureSelect Cancer CGP Assay analysis, TMB is calculated as the ratio of the number of variants detected in a sample to the effective genome coverage in the run. TMB determination is most accurate for samples with coverage ≥1.6 Mb. Runs with 1.0 to 1.6 Mb coverage may generate over-estimated or under-estimated TMB values. Alissa Reporter software does not make TMB determinations for runs with coverage <1 Mb.
- **MSI analysis:** The SureSelect Cancer CGP Assay Probe DNA targets 288 sites available for microsatellite instability (MSI) determinations. Coverage for typical samples is in the range of 250-280 sites, and MSI determination is most accurate for samples having coverage in this range. Samples with coverage in <150 of the available sites may generate over-estimated or under-estimated MSI values. Using Agilent's Alissa Reporter SureSelect Cancer CGP Assay analysis, MSI determinations are not made for samples with coverage of <150 sites.
- **RNA fusions:** The SureSelect Cancer CGP RNA Assay probe design enables detection of RNA fusions in 80 genes, regardless of partner. Agilent's Alissa Reporter software identifies sequencing reads that correspond to both the targeted transcript and a partner transcript (which may or may not also be targeted in the assay). It is possible for these reads to occur in the absence of a fusion event, for example read-through transcription of neighboring genes or through library preparation or sequencing artifacts. Results should be interpreted by trained personnel.
- **RNA exon-skipping:** The SureSelect Cancer CGP RNA assay probe design targets two specific splice variants, EGFRvIII and MET Exon14-skipping. In normal samples, a low-level of variant transcripts may be detected. To minimize false positive calls, use known positive and negative samples for a specific exon skipping locus of interest to define the appropriate threshold for calling the exon skipping event in the selected analysis software. Calls passing the threshold should be further evaluated for variant transcripts per million (TPM) value. A minimum variant TPM of 10 is recommended to indicate sufficient expression of the variant transcript for calling.

6
Appendix 1: SureSelect Cancer CGP Automation

Automation Overview

NGS library preparation for the SureSelect Cancer CGP Assay can be automated using the solutions detailed in Table 44. Review the workflow outline for your automation system on page 60 for the Magnis system or on page 61 for the Bravo system. These sections include links to the relevant SureSelect XT HS2 automation user guides and important tips for optimizing each automation protocol for the SureSelect Cancer CGP Assay.

Table 44 Ordering information for SureSelect Cancer CGP Assay Automation Solutions

Description	Agilent Part Number	Reagent Modules Included				
		Probe	DNA Library Prep + Hyb Reagents	RNA Library Prep + Hyb Reagents	Capture Beads, Purification Beads	Enzymatic DNA Fragmentation
Magnis SureSelect Cancer CGP Assay Kits (32 Samples or 96 Samples)						
Magnis Cancer CGP DNA Reagent Kit, 32 Reactions	G9777A	✓DNA	✓ (Index 1-32)	✕ (not applicable)	✓	✓
Magnis Cancer CGP DNA Reagent Kit, 96 Reactions	G9777B	✓DNA	✓ (Index 1-96 **OR** 97-192)	✕ (not applicable)	✓	✓
Magnis Cancer CGP RNA Reagent Kit, 32 Reactions	G9777C	✓RNA	✕ (not applicable)	✓ (Index 1-32)	✓	✕ (not applicable)
Magnis Cancer CGP RNA Reagent Kit, 96 Reactions	G9777D	✓RNA	✕ (not applicable)	✓ (Index 1-96 **OR** 97-192)	✓	✕ (not applicable)
Agilent Bravo Automation SureSelect Cancer CGP Assay Kits (96 Samples)						
SureSelect Cancer CGP Assay DNA & RNA Kit, 96 Samples Each, Auto	G9966B	✓DNA & RNA†	✓ (Index 1-96)	✓ (Index 97-192)	✓	– (optional; order p/n 5191-4080)
SureSelect Cancer CGP Assay DNA Kit, 96 Samples, Auto	G9967C	✓DNA	✓ (Index 1-96)	✕ (not applicable)	✓	– (optional; order p/n 5191-4080)
SureSelect Cancer CGP Assay RNA Kit, 96 Samples, Auto	G9968C	✓RNA	✕ (not applicable)	✓ (Index 97-192)	✓	✕ (not applicable)

Magnis Automation Workflow

Pre-run instrument and labware preparation

Before you begin, review the appropriate user guide(s) below to familiarize yourself with the automation workflow. Consult the *Materials Required* section and ensure that all materials needed for automated NGS library preparation are available in your laboratory. Verify that your Magnis instrument is equipped with the necessary run protocols and firmware.

Table 45 Magnis automation parameters

SureSelect Cancer CGP Assay	Magnis Automation User Guide Link	Magnis Protocol used for NGS Library Preparation	Magnis Instrument Firmware Version Required
DNA	G9751-90000	*SSEL-DNA-XTHS2-ILM*	v1.3 or later
RNA	G9752-90000	*SSEL-RNA-XTHS2-ILM*	v1.4 or later

Pre-run sample preparation

Prepare and qualify the DNA or RNA samples as directed in the appropriate Magnis SureSelect XT HS2 user guide. All samples for Magnis-automated SureSelect Cancer CGP assays must be prepared in 1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA), using the volumes specified in Table 46.

Table 46 Magnis sample input parameters

Nucleic Acid Input Type	Magnis Protocol used for NGS Library Preparation	Input Amount Options*	Sample Volume Required for Automation Protocol
Unsheared DNA (from high-quality or FFPE samples)	*SSEL-DNA-XTHS2-ILM* **with** enzymatic fragmentation	10 ng, 50 ng, 100 ng, or 200 ng	14 µL
Covaris-sheared DNA (from high-quality or FFPE samples)	*SSEL-DNA-XTHS2-ILM* **without** enzymatic fragmentation	10 ng, 50 ng, 100 ng, or 200 ng	50 µL
Intact RNA or good-quality FFPE RNA samples	*SSEL-RNA-XTHS2-ILM*	10 ng, 50 ng, 100 ng, or 200 ng	10 µL
Poor-quality FFPE RNA samples	*SSEL-RNA-XTHS2-ILM*	50 ng, 100 ng, or 200 ng	10 µL

* Input amounts listed in this table include all options available in the Magnis software for each specific Magnis protocol and input type. The SureSelect Cancer CGP assay is optimized for sample input amounts of 50 ng DNA or RNA. For lower-quality FFPE samples, assay performance may be improved by increasing the amount of DNA or RNA input to 100 ng or 200 ng. Use of 10 ng input DNA or RNA is supported for some Magnis run types, but may lead to reduced performance for the SureSelect Cancer CGP assay.

For optimal SureSelect Cancer CGP assay performance, use of 50 ng input is recommended for most samples. For low-quality FFPE samples, assay performance may be improved by increasing the amount of DNA or RNA input to 100 ng or 200 ng. Some Magnis run types also allow use of 10 ng DNA or RNA input, however use of input <50 ng for the SureSelect Cancer CGP Assay may lead to lower target coverage and reduced detection of low-frequency variants.

All experimental samples processed in the same eight (8)-sample Magnis run should be of the same input amount and same input type.

NOTE

DNA assay runs analyzing FFPE samples can include a high-quality DNA control sample in the same run; select the *Sample Type* option of *FFPE DNA* during run setup for these runs.

For RNA assay runs analyzing FFPE samples, any control intact RNA samples must be processed in a separate Magnis run with *Intact RNA* selected as *Sample Type* during run setup. Intact RNA samples are not properly fragmented under FFPE RNA run conditions.

Performing the Magnis NGS library preparation run

Perform automated NGS library preparation as directed in the appropriate Magnis user guide, through the final library quantification step.

Post-run library processing

Follow the post-run library processing guidelines provided in this publication, starting with pooling the quantified NGS libraries for multiplexed sequencing, as outlined on page 49. Use the NGS support resources in this publication for the Magnis-processed libraries (page 50 to page 57 and index sequences provided on page 79 to page 83).

Bravo Automation Workflow

Before you begin, review the appropriate user guide(s) below to familiarize yourself with the automation workflows. Consult the *Materials Required* section and ensure that all materials needed for Bravo-automated NGS library preparation are available in your laboratory. Verify that your Agilent NGS Workstation or Bravo instrument is equipped with the required VWorks software forms.

Table 47 Bravo automation summary

SureSelect Cancer CGP Assay	Bravo Automation User Guide Link		VWorks Software Form
	NGS Workstation (Option B)*	NGS Bravo (Option A)	
DNA	G9985-90010	G9985-90020	SureSelect XT HS2 DNA Form
RNA	G9993-90010	G9993-90020	SureSelect XT HS2 RNA Form

* The Bravo NGS Workstation Option B+, with an on-deck thermal cycler, is also available with protocols for processing SureSelect XT HS2 DNA assays. See publication G9985-90015 for protocol details. The provided SureSelect XT HS2 DNA assay protocols can be used for processing SureSelect Cancer CGP Assay samples, after verification of the required performance in your laboratory (see Table 50 on page 65 for PCR cycle numbers and other run attributes). At the time of this publication, SureSelect XT HS2 RNA assay automation protocols are not available for the Bravo NGS Workstation Option B+.

Pre-run sample preparation and qualification

Prepare, qualify, and quantify total RNA and gDNA samples according to the instructions provided in this publication (see Table 48 for links to the appropriate sections). For workflows that include mechanical DNA shearing, also use the instructions provided on page 27 of this publication for the fragmentation workflow segment.

Samples must be placed in the appropriate Bravo-compatible 96-well plate for further processing, using the volumes and solvents specified in Table 48. For optimal SureSelect Cancer CGP assay performance, use of 50 ng input is recommended for most samples. For low-quality FFPE samples, assay performance may be improved by increasing the amount of DNA or RNA input to up to 200 ng. Runs may be set up using as little as 10 ng DNA or RNA input, with possible negative impacts on target coverage and detection of low-frequency variants.

Table 48 Bravo automation sample input parameters

SureSelect Cancer CGP Assay	Fragmentation Method	Link to Sample Prep/QC Steps	Sample Composition Required to Begin Automation
RNA	Bravo-automated chemical fragmentation (only high-quality intact RNA samples require fragmentation; see Table 49 on page 63)	page 17 to page 18	10 to 200 ng* total RNA in 10 µL nuclease-free water (starting sample for VWorks protocol *Fragmentation_XT_HS2_RNA*)
DNA	Bravo-automated enzymatic fragmentation (see Table 50 on page 65)	page 24 to page 25	10 to 200 ng* gDNA in 15 µL nuclease-free water (starting sample for VWorks protocol *EnzFrag_XT_HS2_ILM*)
	Mechanical shearing (non-automated)	page 24 to page 25 and page 27 to page 28	10 to 200 ng* DNA fragments in 50 µL 1X Low TE Buffer (starting sample for VWorks runset *LibraryPrep_XT_HS2_ILM*)

* Follow the input amount recommendations provided in this publication (use ≥50 ng DNA or RNA input, based on sample quality, for optimal performance). Runs may be set up using as little as 10 ng DNA or RNA input for automated processing using SureSelect XT HS2 chemistry, with possible negative impacts on target coverage and detection of low-frequency variants.

All experimental samples processed in the same plate should be of the same input type and input amount to allow amplification and fragmentation, where applicable, under the same conditions.

NOTE

RNA assay runs analyzing FFPE samples can include high-quality RNA control samples on the same plate, with the run modifications listed on page 64.

DNA assay runs analyzing FFPE samples can include high-quality DNA control samples on the same plate, with the run modifications listed on page 66.

Performing the NGS library preparation run

For a summary of the VWorks protocols used for SureSelect Cancer CGP automation, see Table 49 on page 63 for the RNA Assay and see Table 50 on page 65 for the DNA Assay.

To set up and run each VWorks automation protocol, use the detailed instructions provided in the SureSelect XT HS2 automation user guides listed in Table 47 on page 61.

RNA and DNA samples are processed separately through the Bravo automation protocols, using VWorks protocols initiated from the VWorks Form corresponding to the DNA or RNA sample type.

RNA Assay automation protocols

Table 49 RNA Assay--overview of VWorks automation protocols and runsets

Workflow Step	Substep	VWorks Protocols* Used for Agilent NGS Workstation Automation	Notes
AMPure XP Bead Aliquoting	For use in Second-Strand Synthesis runset	AMPureXP_Aliquot (Case Second-Strand)	You can prepare all AMPure XP bead plates needed for same-day use at the start of the day's workflow to reduce delays between steps. Keep prepared plates at 4°C for up to 24 hours.
	For use in Library Prep runset	AMPureXP_Aliquot (Case Library Prep)	
	For use in Pre-Capture PCR purification protocol	AMPureXP_Aliquot (Case Pre-Capture PCR)	
	For use in Post-Capture PCR purification protocol	AMPureXP_Aliquot (Case Post-Capture PCR)	
RNA Preparation and cDNA Conversion	Mix RNA samples with the 2X Priming Buffer	Fragmentation_XT_HS2_RNA	Process intact RNA and FFPE RNA assays on separate plates. Do the 94°C fragmentation step only for intact RNA samples. If intact control included on FFPE sample plate, see page 64.
	Synthesize first-strand cDNA	FirstStrandcDNA_XT_HS2_RNA	
	Synthesize and purify second-strand cDNA	SecondStrand_XT_HS2_RNA	
Library Preparation	Prepare and purify molecular-barcoded DNA libraries	Runset LibraryPrep_XT_HS2_ILM	—
	Amplify DNA libraries with dual indexing primer pairs	Pre-CapPCR_XT_HS2_ILM	Use 12 PCR cycles for high-quality RNA input or 15 PCR cycles for FFPE RNA input. If intact RNA control was included in FFPE sample run, see page 64.
	Purify indexed DNA libraries using AMPure XP beads	AMPureXP_XT_HS2_ILM (Case Pre-Capture PCR – SinglePlex)	Use instructions for single-plex hyb/post-capture pooling
	Set up plates for library QC using Agilent TapeStation platform	TS_D1000	May also be performed manually (see page 37)
Hybridization and Capture	Aliquot 200 ng of prepped cDNA libraries into hybridization plate	Aliquot_Libraries	Use instructions for single-plex hyb; may also be performed manually (see page 40)
	Hybridize prepped libraries (target enrichment)	Hyb_XT_HS2_ILM	Use instructions for probes ≥3 Mb, single probe in all rows
	Capture and wash DNA hybrids	Runset SSELCapture&Wash_XT_HS2	—
Post-Capture Sample Processing	Amplify target-enriched libraries	Post-CapPCR_XT_HS2_ILM	Use 13 PCR cycles
	Purify enriched, amplified libraries using AMPure XP beads	AMPureXP_XT_HS2_ILM (Case Post-Capture PCR)	—
	Set up plates for final library QC using Agilent TapeStation platform	TS_HighSensitivity_D1000	May also be performed manually (see page 46)

* Use the SureSelect XT HS2 **RNA** VWorks form to open each automation protocol. Some of the protocols and runsets accessed from the SureSelect XT HS2 DNA Form are not compatible with the reagent and labware positioning specifications used for the RNA assay.

Running intact RNA controls with FFPE RNA samples

- Perform the *Fragmentation_XT_HS2_RNA* protocol liquid-handling steps as directed in the Bravo automation user guide, starting with all samples on the same plate. Once complete, remove the plate from the Bravo deck to ice, then transfer the intact control RNA sample(s) to well(s) of a fresh strip tube. Perform the 94°C thermal cycler incubation step described in the Bravo automation user guide using only the intact control RNA strip. Once complete, transfer each fragmented control sample back to its original FFPE RNA sample plate well for further processing.
- Perform the *Pre-CapPCR_XT_HS2_ILM* protocol liquid-handling steps as directed in the Bravo automation user guide, starting with all samples on the same plate. Once complete, remove the plate from the Bravo deck to ice, then transfer the control RNA library amplification reaction(s) to well(s) of a fresh strip tube. Amplify the FFPE and control sample libraries using separate thermal cyclers using the amplification cycle number appropriate for each sample type. Once the thermal cycling programs are complete, transfer each control library from the control strip back to its original FFPE RNA sample plate well for further processing.

DNA Assay automation protocols

Table 50 DNA Assay--overview of VWorks automation protocols and runsets

Workflow step	Substep	VWorks Protocols* Used for Agilent NGS Workstation Automation	Notes
AMPure XP Bead Aliquoting	For use in Library Prep runset	AMPureXP_Aliquot (Case Library Prep)	You can prepare all AMPure XP bead plates needed for same-day use at the start of the day's workflow to reduce delays between steps. Keep prepared plates at 4°C for up to 24 hours.
	For use in Pre-Capture PCR purification protocol	AMPureXP_Aliquot (Case Pre-Capture PCR)	
	For use in Post-Capture PCR purification protocol	AMPureXP_Aliquot (Case Post-Capture PCR)	
Enzymatic DNA Fragmentation	Shear DNA samples using enzymatic fragmentation	EnzFrag_XT_HS2_ILM	Process intact DNA and FFPE DNA assays on separate plates. If intact control included in FFPE DNA assay, see page 66. Enzymatic DNA fragmentation may be replaced with Covaris DNA shearing using manual liquid handing and shearing steps (see page 27).
	Dilute fragmented samples to appropriate concentration	EnzFrag_Dil_XT_HS2_ILM	
Library Preparation	Prepare and purify molecular-barcoded DNA libraries	Runset LibraryPrep_XT_HS2_ILM	—
	Amplify DNA libraries with dual indexing primer pairs	Pre-CapPCR_XT_HS2_ILM	Use 9 PCR cycles for high-quality DNA input or 12 PCR cycles for FFPE DNA input. If intact DNA control was included in FFPE sample run, see page 66.
	Purify indexed DNA libraries using AMPure XP beads	AMPureXP_XT_HS2_ILM (Case Pre-Capture PCR – SinglePlex)	Use instructions for single-plex hyb/post-capture pooling
	Set up plates for library QC using Agilent TapeStation platform	TS_D1000	May also be performed manually (see page 37)
Hybridization and Capture	Aliquot 500-1000 ng of prepped libraries into hybridization plate	Aliquot_Libraries	Use instructions for single-plex hyb; may also be performed manually (see page 40)
	Hybridize prepped libraries (target enrichment)	Hyb_XT_HS2_ILM	Use instructions for probes ≥3 Mb, single probe in all rows
	Capture and wash DNA hybrids	Runset SSELCapture&Wash_XT_HS2	—
Post-Capture Sample Processing	Amplify target-enriched libraries	Post-CapPCR_XT_HS2_ILM	Use 13 PCR cycles
	Purify enriched, amplified libraries using AMPure XP beads	AMPureXP_XT_HS2_ILM (Case Post-Capture PCR)	—
	Set up plates for final library QC using Agilent TapeStation platform	TS_HighSensitivity_D1000	May also be performed manually (see page 46)

* Use the SureSelect XT HS2 **DNA** VWorks form to open each automation protocol. Some of the protocols and runsets accessed from the SureSelect XT HS2 RNA Form are not compatible with the reagent and labware positioning specifications required for the DNA assay.

Running intact DNA controls with FFPE DNA samples

- For workflows including enzymatic fragmentation, perform the *EnzFrag_XT_HS2_ILM* protocol liquid-handling steps as directed in the Bravo automation user guide with all samples on the same plate. Once complete, all samples can be fragmented together, using the thermal cycler program for FFPE DNA samples provided in the Bravo automation user guide. After fragmentation, resume the workflow for the sample plate with the *EnzFrag_Dil_XT_HS2_ILM* protocol.
- For workflows including mechanical shearing, use the shearing conditions specified for each sample type in this publication (see page 27 to page 28). Once complete, all sheared DNA samples can be placed on the same plate for further automated processing, starting with the *LibraryPrep_XT_HS2_ILM* runset.
- Perform the *Pre-CapPCR_XT_HS2_ILM* protocol liquid-handling steps as directed in the Bravo automation user guide, starting with all samples on the same plate. Once complete, remove the plate from the Bravo deck to ice, then transfer the control DNA library amplification reaction(s) to well(s) of a fresh strip tube. Amplify the FFPE and control sample libraries on separate thermal cyclers using the amplification cycle number appropriate for each sample type. Once the thermal cycling programs are complete, transfer each control library from the control strip back to its original FFPE DNA sample plate well for further processing.

Post-run library processing

Follow the post-run library processing guidelines provided in this publication, starting with pooling the quantified NGS libraries for multiplexed sequencing, as outlined on page 49. Use the NGS support resources in this publication for the Magnis-processed libraries (page 50 to page 57 and index sequences provided on page 79 to page 83).

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Appendix 2: SureSelect Cancer Tumor-Specific Assays

Overview of SureSelect Cancer Tumor-Specific Assays

SureSelect Cancer Tumor-Specific Assays have been developed for several specific solid tumor types, in order to interrogate a variety of genomic features including SNVs, Indels, CNVs and translocations at key loci for each tumor type. See the Cancer NGS Assays page at Agilent.com for the most current list of tumor-specific assays available and for additional details on the genomic features interrogated by each of the SureSelect Cancer Assay Probes.

Running the SureSelect Cancer Assay requires separate purchase of the relevant SureSelect Cancer Assay Probe and a SureSelect XT HS2 DNA Reagent Kit. Target-enriched NGS libraries are prepared from gDNA samples using this set of components using the protocols provided in this publication with the minor modifications detailed in this Appendix. Once sequencing data is collected for the assay samples, analysis is performed using the appropriate NGS analysis software tool(s) for the variant discovery goals of your research.

Materials Required for SureSelect Cancer Tumor-Specific Assays

Running the SureSelect Cancer Assay requires the components listed below:

- SureSelect Cancer Assay DNA Probe (see Table 51 for a list of probes available at the time of this publication)
- SureSelect XT HS2 DNA Reagent Kit with Fast-Hyb, Post-capture pooling target enrichment reagents (seeTable 52 on page 68 for a list of compatible reagent kits)
- Additional reagents and equipment required for DNA assays using the selected sample type detailed in Table 2 on page 12 through Table 4 on page 14

Table 51 SureSelect Cancer Assay Probes–Select One

Assay	Probe Design ID	Agilent Part Number	
		16 Hybs	96 Hybs*
SureSelect Cancer Lung Assay Probe, DNA	A3464871	p/n 5282-0060	p/n 5282-0061
SureSelect Cancer Colon Assay Probe, DNA	A3464881	p/n 5282-0062	p/n 5282-0063
SureSelect Cancer Pancreas Assay Probe, DNA	A3464891	p/n 5282-0064	p/n 5282-0065
SureSelect Cancer Kidney Assay Probe, DNA	A3464901	p/n 5282-0066	p/n 5282-0067
SureSelect Cancer Bladder Assay Probe, DNA	A3464911	p/n 5282-0068	p/n 5282-0069

* Compatible with both manual-processing and Bravo-automated processing of 96 samples.

Table 52 Ordering Information for Compatible SureSelect XT HS2 DNA Kits

Agilent Part Number	Samples Processed	Library Prep Kit (Index Pairs Included)	Target Enrichment Kit	Capture Beads, Purification Beads	Enzymatic DNA Fragmentation Kit
Complete SureSelect XT HS2 DNA Starter Kit (16 Samples)					
G9982A	16*	✓ Index 1–16	✓ (Fast-Hyb, Post-Cap Pool)	✓	✓
SureSelect XT HS2 DNA Reagent Kits with AMPure® XP† & Streptavidin Beads (96 Samples)					
G9984A	96‡	✓ Index 1–96	✓ (Fast-Hyb, Post-Cap Pool)	✓	Optional (order Agilent p/n 5191-4080 for manual processing or p/n 5191-6764 for Bravo-automation)
G9984B	96‡	✓ Index 97–192			
G9984C	96‡	✓ Index 193–288			
G9984D	96‡	✓ Index 289–384			
SureSelect XT HS2 DNA Reagent Kits					
G9981A	16*	✓ Index 1–16	✓ (Fast-Hyb, Post-Cap Pool)	✖ Requires separate purchase of materials below: • Dynabeads MyOne Streptavidin T1 from Thermo Fisher Scientific • AMPure® XP Kit from Beckman Coulter	Optional (order Agilent p/n 5191-4080 for manual processing or p/n 5191-6764 for Bravo-automation)
G9983A	96‡	✓ Index 1–96			
G9983B	96‡	✓ Index 97–192			
G9983C	96‡	✓ Index 193–288			
G9983D	96‡	✓ Index 289–384			

* Kits are compatible with manual-processing of 16 samples using the protocols and supported run sizes described in this publication.

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

‡ Kits are compatible with manual-processing of 96 samples using the protocols and supported run sizes described in this publication. Kits are also compatible with Bravo-automated processing of 96 samples using the supported run configurations described in the Bravo automation user guides (see Table 47 on page 61).

Running the SureSelect Cancer Tumor-Specific Assay

- Consult "Sample requirements" on page 9 before you begin.
- Prepare, qualify, and fragment gDNA samples as directed on page 24 to page 28.
- Prepare SureSelect XT HS2 libraries as directed on page 30 to page 37, using the instructions specific for DNA samples.
- Target-enrich the libraries using the appropriate SureSelect Cancer Probe. Follow the instructions provided on page 39 to page 46, with the following modification. In the post-capture PCR thermal cycler program in Table 33 on page 43, amplify using 16 PCR cycles (replacing 13 cycles) in segment 2.
- Process the target-enriched libraries for NGS as described on page 48 to page 52, with the following modification. As a replacement for the sequencing depth recommendations provided on page 48, Agilent recommends using ≥5M reads per sample for SureSelect Cancer Colon, Kidney, and Bladder Assays or ≥7.5M reads per sample for SureSelect Cancer Lung and Pancreas Assays.
- The SureSelect Cancer Assay probes are compatible with processing using Agilent's Magnis and Bravo automation systems as detailed below. Performance specifications have not, however, been specifically verified for libraries enriched using these automation systems.
- Magnis automation is available for the SureSelect Cancer Assay probe designs when the appropriate SureSelect Cancer Assay Probe Design ID (see Table 51 on page 67) is used to order a custom Magnis SureSelect XT HS2 DNA Reagent Kit. Visit Agilent's SureDesign site for custom Magnis probe design and kit ordering information. See page 60 to page 61 for additional Magnis automation guidelines. Alternatively, Magnis automation can be completed using the probe products listed in Table 51 on page 67 in conjunction with Magnis SureSelect XT HS2 DNA (No Probe) Reagent Kits (Agilent P/N G9750B), provided with empty probe input strips which can be filled with the appropriate SureSelect Cancer Assay probe(s). See the Magnis SureSelect XT HS2 DNA user guide for more information on use of self-filled probe strips with the Magnis automation protocol *SSEL-DNA-XTHS2-EPIS-ILM*.
- For Bravo automation of the SureSelect Cancer Assays, use the instructions provided on page 61 to page 62 and page 65 to page 66 with the following modification. When running the *Post-CapPCR_XT_HS2_ILM* Bravo protocol listed in Table 50 on page 65, use 16 PCR cycles (replacing 13 cycles). The 96-Hyb SureSelect Cancer Assay Probes listed in Table 51 on page 67, along with the 96-Sample SureSelect XT HS2 DNA Reagent Kits listed in Table 52 on page 68 are compatible with Bravo-automated processing.
- For co-analysis of RNA transcriptome variants in samples analyzed using a SureSelect Cancer assay, Agilent recommends analyzing the samples using the SureSelect Cancer CGP RNA probe in parallel with the selected SureSelect Cancer Assay DNA probe. See page 8 for a description of the variants targeted by the SureSelect Cancer CGP RNA Assay and see Table 59 on page 77 for CGP RNA probe ordering information.

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Appendix 3: SureSelect Cancer Custom Panel Assays

Overview of SureSelect Cancer Custom Panel Assays

SureSelect Cancer Custom Panels can include content from the pre-defined SureSelect Cancer CGP DNA Assay panel or SureSelect Cancer Tumor-Specific Assay panels, along with user-defined content. The dedicated design tool in SureDesign provides a seamless customization process for including or excluding specific targets in the existing panels along with adding content not found in existing panels. To begin the SureSelect Cancer Custom Panel design process, visit Agilent's SureDesign site.

Running the assay requires separate purchase of a SureSelect Cancer Custom Panel and a SureSelect XT HS2 DNA Reagent Kit. Target-enriched NGS libraries are prepared from gDNA samples with this set of components using the protocols provided in this publication with the minor modifications detailed in this Appendix. Once sequencing data is collected for the assay samples, analysis is performed using the appropriate NGS analysis software tool(s) for the variant discovery goals of your research.

Materials Required for SureSelect Cancer Custom Panel Assays

Running the assay requires the components listed below:

- SureSelect Cancer Custom Panel probe. See Table 53 for a list of user-customized probes available from SureDesign at the time of this publication. The SureSelect Cancer Custom Panel probes are also available for Magnis automation; see page 72 for more information.
- SureSelect XT HS2 DNA Reagent Kit with Fast-Hyb, Post-capture pooling target enrichment reagents (seeTable 54 on page 71 for a list of compatible reagent kits)
- Additional reagents and equipment required for DNA assays using the selected sample type detailed in Table 2 on page 12 through Table 4 on page 14

Table 53 SureSelect Cancer Custom Panel options

Custom Design Type	Agilent Part Number		
	16 Hybs	96 Hybs	96 Hybs Auto*
SureSelect Cancer CGP Catalog + Tier 1 Custom	p/n 5282-0170	p/n 5282-0171	p/n 5282-0172
SureSelect Cancer Custom Tier 1	p/n 5282-0164	p/n 5282-0165	p/n 5282-0166
SureSelect Cancer Custom Tier 2	p/n 5282-0167	p/n 5282-0168	p/n 5282-0169

* Compatible with Bravo-automated processing of 96 samples.

Table 54 Ordering Information for Compatible SureSelect XT HS2 DNA Kits

Agilent Part Number	Samples Processed	Library Prep Kit (Index Pairs Included)	Target Enrichment Kit	Capture Beads, Purification Beads	Enzymatic DNA Fragmentation Kit
Complete SureSelect XT HS2 DNA Starter Kit (16 Samples)					
G9982A	16*	✓ Index 1–16	✓ (Fast-Hyb, Post-Cap Pool)	✓	✓
SureSelect XT HS2 DNA Reagent Kits with AMPure® XP† & Streptavidin Beads (96 Samples)					
G9984A	96‡	✓ Index 1–96	✓ (Fast-Hyb, Post-Cap Pool)	✓	Optional (order Agilent p/n 5191-4080 for manual processing or p/n 5191-6764 for Bravo-automation)
G9984B	96‡	✓ Index 97–192			
G9984C	96‡	✓ Index 193–288			
G9984D	96‡	✓ Index 289–384			
SureSelect XT HS2 DNA Reagent Kits					
G9981A	16*	✓ Index 1–16	✓ (Fast-Hyb, Post-Cap Pool)	✖ Requires separate purchase of materials below: • Dynabeads MyOne Streptavidin T1 from Thermo Fisher Scientific • AMPure® XP Kit from Beckman Coulter	Optional (order Agilent p/n 5191-4080 for manual processing or p/n 5191-6764 for Bravo-automation)
G9983A	96‡	✓ Index 1–96			
G9983B	96‡	✓ Index 97–192			
G9983C	96‡	✓ Index 193–288			
G9983D	96‡	✓ Index 289–384			

* Kits are compatible with manual-processing of 16 samples using the protocols and supported run sizes described in this publication.

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

‡ Kits are compatible with manual-processing of 96 samples using the protocols and supported run sizes described in this publication. Kits are also compatible with Bravo-automated processing of 96 samples using the supported run configurations described in the Bravo automation user guides (see Table 47 on page 61).

Running a SureSelect Cancer Custom Panel Assay

Prepare NGS libraries from gDNA samples using the protocols provided in this publication with the minor modifications detailed here.

- Consult "Sample requirements" on page 9 before you begin.
- Prepare, qualify, and fragment gDNA samples as directed on page 24 to page 28.
- Prepare SureSelect XT HS2 libraries as directed on page 30 to page 37, using the instructions specific for DNA samples.
- Target-enrich the libraries using the SureSelect Cancer Custom Panel probe. Follow the instructions provided on page 39 to page 46, with the following modification. In the post-capture PCR thermal cycler program in Table 33 on page 43 (Segment 2), amplify using the PCR cycle number appropriate for the custom design size (see Table 55 for guidelines). Cycle numbers in Table 55 are estimates to give yield of 10–20 nM; adjustment may be necessary based on the specific probe design and sample type/quality used in the assay.

Table 55 Post-capture PCR cycle number recommendations

Design Size	Post-capture PCR Cycles
Probes <500 kb	16 cycles
Probes 0.5–2 Mb	14 cycles
Probes 2–3 Mb	13 cycles
Probes 3–6 Mb	12 cycles
Probes >6 Mb	11 cycles

- Process the target-enriched libraries for NGS as described on page 48 to page 52, with the following modification. As a replacement for the sequencing depth recommendations provided on page 48, Agilent recommends targeting a raw sequencing depth of ≥3000X reads per sample. The recommended minimum raw reads for a specific design size is calculated as follows for paired-end sequencing:

  Million reads = [(Mb design size*3000)/sequencing read length]

- SureSelect Cancer Custom Panel Assays are compatible with processing using Agilent's Magnis and Bravo automation systems as detailed below.
- Magnis automation is available for the SureSelect Cancer Custom Panel Assays. Contact your local Agilent representative for assistance with ordering Magnis SureSelect XT HS2 DNA Reagent Kits with SureSelect Cancer Custom Panel probe strips. Alternatively, Magnis automation can be completed using the probe products listed in Table 53 on page 70 in conjunction with Magnis SureSelect XT HS2 DNA (No Probe) Reagent Kits (Agilent P/N G9750B), provided with empty probe input strips which can be filled with the appropriate SureSelect Cancer Custom Panel probe(s). See the Magnis SureSelect XT HS2 DNA user guide for more information on use of self-filled probe strips with the Magnis automation protocol *SSEL-DNA-XTHS2-EPIS-ILM*. See page 60 to page 61 for additional Magnis automation guidelines.

- For Bravo automation of the SureSelect Cancer Custom Panel Assays, use the instructions provided on page 61 to page 62 and page 65 to page 66 with the following modification. When running the *Post-CapPCR_XT_HS2_ILM* Bravo protocol listed in Table 50 on page 65, amplify using the PCR cycle number appropriate for the custom design size (see Table 55 on page 72 for guidelines). Cycle numbers in Table 55 are estimates to give yield of 10–20 nM; adjustment may be necessary based on the specific probe design and sample type/quality used in the assay. See Table 53 on page 70 for Bravo-automation compatible probe part numbers. The 96-Sample SureSelect XT HS2 DNA Reagent Kits listed in Table 54 on page 71 are compatible with Bravo-automated processing.
- For co-analysis of RNA transcriptome variants in samples analyzed for genome variants using a SureSelect Cancer Custom Panel Assay, Agilent recommends analyzing the samples using the SureSelect Cancer CGP RNA probe in parallel with the SureSelect Cancer Custom Panel probe. See page 8 for a description of the variants targeted by the SureSelect Cancer CGP RNA Assay and see Table 59 on page 77 for CGP RNA probe ordering information.

9 Reference

This section contains reference information, including Reagent Kit contents, index sequences, and troubleshooting information for the SureSelect Cancer CGP Assay.

Reagent Kit Contents

SureSelect Cancer CGP Assay Kits include the component kits listed in Table 56 (for DNA + RNA kits), Table 57 (for DNA only kits), and Table 58 (for RNA only kits). Detailed contents of each of the multi-part component kits are shown in Table 59 through Table 65 on the following pages.

Table 56 Contents of SureSelect Cancer CGP Assay Kits for DNA + RNA analysis

Component Kit Name	Storage Condition	Component Kit Part Number		Usage
		G9965A DNA+RNA Starter Kit (16 Samples Each)	G9966A DNA+RNA Kit (96 Samples Each)	
SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0146	5500-0147	DNA Library Prep
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	–20°C	5191-5687 (Index Pairs 1–16)	5191-5688 (Index Pairs 1–96)	DNA Library Prep
SureSelect cDNA Module (Pre PCR)	–20°C	5500-0148	5500-0149	RNA Library Prep
SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0150	5500-0151	RNA Library Prep
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	–20°C	5191-6971 (Index Pairs 17–32)	5191-5689 (Index Pairs 97–192)	RNA Library Prep
SureSelect Cancer CGP Assay Probes, DNA & RNA	–80°C	5191-6990	5191-6991	DNA and RNA Library Enrichment
SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	Room Temperature	5190-9685 (2 kits)	5190-9687 (2 kits)	DNA and RNA Library Enrichment
SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	–20°C	5191-6686 (2 kits)	5191-6688 (2 kits)	DNA and RNA Library Enrichment
SureSelect Streptavidin Beads	+4°C	5191-5741 (2 vials)	5191-5742 (2 vials)	DNA and RNA Library Enrichment
SureSelect DNA AMPure® XP Beads*	+4°C	5191-5739	5191-5740	DNA Library Prep/Enrichment Purifications
SureSelect RNA AMPure® XP Beads*	+4°C	5191-6670	5191-6671	RNA Library Prep/Enrichment Purifications
SureSelect Enzymatic Fragmentation Kit	–20°C	5191-4079	Not supplied, optional (order p/n 5191-4080 separately)	DNA Library Prep
OneSeq Human Reference DNA, Female	+4°C	5190-8850	Not supplied, optional (order p/n 5190-8850 separately)	Control and unmatched reference DNA
QPCR Human Reference Total RNA	–80°C	750500	Not supplied, optional (order p/n 750500 separately)	Control RNA

* AMPure, Beckman, and Beckman Coulter are trademarks or registered trademarks of Beckman Coulter, Inc. SureSelect DNA AMPure XP Beads and SureSelect RNA AMPure XP Beads may be used interchangeably.

Table 57 Contents of SureSelect Cancer CGP Assay Kits for DNA analysis

Component Kit Name	Storage Condition	Component Kit Part Number		Usage
		G9967A DNA Kit (16 Samples)	G9967B DNA Kit (96 Samples)	
SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0146	5500-0147	DNA Library Prep
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	–20°C	5191-5687 (Index Pairs 1–16)	5191-5688 (Index Pairs 1–96)	DNA Library Prep
SureSelect Cancer CGP Assay Probe, DNA	–80°C	5280-0035	5280-0036	Target Enrichment
SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	Room Temperature	5190-9685	5190-9687	Target Enrichment
SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	–20°C	5191-6686	5191-6688	Target Enrichment
SureSelect Streptavidin Beads	+4°C	5191-5741	5191-5742	Target Enrichment Capture
SureSelect DNA AMPure® XP Beads	+4°C	5191-5739	5191-5740	DNA Library Prep/ Enrichment Purifications

Table 58 Contents of SureSelect Cancer CGP Assay Kits for RNA analysis

Component Kit Name	Storage Condition	Component Kit Part Number		Usage
		G9968A RNA Kit (16 Samples)	G9968B RNA Kit (96 Samples)	
SureSelect cDNA Module (Pre PCR)	–20°C	5500-0148	5500-0149	RNA Library Prep
SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0150	5500-0151	RNA Library Prep
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	–20°C	5191-6971(Index Pairs 17–32)	5191-5689 (Index Pairs 97–192)	RNA Library Prep
SureSelect Cancer CGP Assay Probe, RNA	–80°C	5191-6996	5191-6997	Target Enrichment
SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	Room Temperature	5190-9685	5190-9687	Target Enrichment
SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	–20°C	5191-6686	5191-6688	Target Enrichment
SureSelect Streptavidin Beads	+4°C	5191-5741	5191-5742	Target Enrichment Capture
SureSelect RNA AMPure® XP Beads	+4°C	5191-6670	5191-6671	RNA Library Prep/ Enrichment Purifications

Table 59 Contents of SureSelect Cancer CGP Assay Probe Kits

Kit Part Number	Product Name	Storage Condition	Component(s) Provided	Usage
5191-6990	SureSelect Cancer CGP Assay Probes, DNA & RNA, 16 Hyb Reactions/Probe	–80°C	5264-1001 SureSelect Cancer CGP Assay Probe DNA	DNA Library Enrichment
			5191-6894 SureSelect Cancer CGP Assay Probe RNA	RNA Library Enrichment
5191-6991	SureSelect Cancer CGP Assay Probes, DNA & RNA, 96 Hyb Reactions/Probe	–80°C	5264-1002 SureSelect Cancer CGP Assay Probe DNA	DNA Library Enrichment
			5191-6896 SureSelect Cancer CGP Assay Probe RNA	RNA Library Enrichment
5280-0035	SureSelect Cancer CGP Assay Probe, DNA, 16 Hyb Reactions	–80°C	5264-1001 SureSelect Cancer CGP Assay Probe DNA	DNA Library Enrichment
5280-0036	SureSelect Cancer CGP Assay Probe, DNA, 96 Hyb Reactions	–80°C	5264-1002 SureSelect Cancer CGP Assay Probe DNA	DNA Library Enrichment
5191-6996	SureSelect Cancer CGP Assay Probe, RNA, 16 Hyb Reactions	–80°C	5191-6894 SureSelect Cancer CGP Assay Probe RNA	RNA Library Enrichment
5191-6997	SureSelect Cancer CGP Assay Probe, RNA, 96 Hyb Reactions	–80°C	5191-6896 SureSelect Cancer CGP Assay Probe RNA	RNA Library Enrichment

Table 60 SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR) content

Kit Component	16 Reaction Kit (p/n 5500-0146)	96 Reaction Kit (p/n 5500-0147)
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 61 SureSelect cDNA Module (Pre PCR) content

Kit Component	16 Reaction Kit (p/n 5500-0148)	96 Reaction Kit (p/n 5500-0149)
2X Priming Buffer	tube with purple cap	tube with purple cap
First Strand Master Mix*	amber tube with amber cap	amber tube with amber cap
Second Strand Enzyme Mix	tube with blue cap	bottle
Second Strand Oligo Mix	tube with yellow cap	tube with yellow cap

* The First Strand Master Mix contains actinomycin D. Keep the reagent in the supplied amber vial to protect the contents from exposure to light.

Table 62 SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR) content

Kit Component	16 Reaction Kit (p/n 5500-0150)	96 Reaction Kit (p/n 5500-0151)
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
XT HS2 RNA Adaptor Oligo Mix	tube with green cap	tube with green 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 63 SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) content

Kit Component	16 Reaction Kits*		96 Reaction Kits†	
	p/n 5191-5687 (use for DNA libraries)	p/n 5191-6971 (use for RNA libraries)	p/n 5191-5688 (use for DNA libraries)	p/n 5191-5689 (use for RNA libraries)
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)	Black 8-well strip tube (index pairs 17-24) AND red 8-well strip tube (index pairs 25-32)	Orange 96-well plate (index pairs 1–96)	Blue 96-well plate (index pairs 97–192)

* See page 79 through page 80 for index pair sequence information; see page 84 for index strip position maps.

† See page 79 through page 83 for index pair sequence information; see page 85 for index plate position maps.

Table 64 SureSelect Target Enrichment Kit, ILM Hyb Module Box 1 (Post PCR) content

Kit Component	16 Reaction Kit (p/n 5190-9685)	96 Reaction Kit (p/n 5190-9687)
SureSelect Binding Buffer	bottle	bottle
SureSelect Wash Buffer 1	bottle	bottle
SureSelect Wash Buffer 2	bottle	bottle

Table 65 SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR) content

Kit Component	16 Reaction Kit (p/n 5191-6686)	96 Reaction Kit (p/n 5191-6688)
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. One primer pair is provided in each well of 8-well strip tubes (16 reaction kits; see Figure 7 for a map) or of 96-well plates (96 reaction kits; see page 85 for plate maps). Each well contains a single-use aliquot of a specific pair of forward plus reverse primers.

The nucleotide sequence of the index portion of each primer is provided in Table 67 on page 80 through Table 70 on page 83. Index sequences can also be obtained by downloading the SureSelect XT HS2 Index Sequence Resource Excel spreadsheet from Agilent.com.

NOTE

Clicking the Excel spreadsheet link from Agilent.com automatically downloads the index spreadsheet to the default folder for downloaded files saved by your web browser. Locate the file in the folder and open it in Microsoft Excel or another compatible spreadsheet program. The first tab of the spreadsheet provides instructions for use of the spreadsheet contents.

In Table 67 through Table 70 and in the downloadable Excel spreadsheet, P7 indexes are shown in forward orientation, applicable to any of the supported Illumina platforms. P5 indexes are shown in two orientations (forward and reverse complement) for use with different platforms and sequencing run setup and management tools, e.g., Local Run Manager and Instrument Run Setup. Illumina sequencing platforms and their P5 sequencing orientation are shown in Table 66. Correct representation of the P5 index orientation in sample sheets or during sequencing run setup is crucial to successful demultiplexing. Refer to Illumina support documentation and resources to determine the correct P5 index orientation for your application.

Table 66 P5 index sequencing orientation by Illumina platform

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

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

SureSelect XT HS2 Index Primer Pair Sequences

Table 67 SureSelect XT HS2 Index Primer Pairs 1–48, provided in orange 96-well plate or in strip tubes

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

Table 68 **SureSelect XT HS2 Index Primer Pairs 49–96, provided in orange 96-well plate**

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

Table 69 **SureSelect XT HS2 Index Primer Pairs 97–144, provided in blue 96-well plate**

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

Table 70 **SureSelect XT HS2 Index Primer Pairs 145–192, provided in blue 96-well plate**

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

Index Primer Pair Strip Tube and Plate Maps

SureSelect XT HS2 Index Primer Pairs 1-16 and 17-32 (provided with 16 reaction kits) are supplied in sets of two 8-well strip tubes as detailed below.

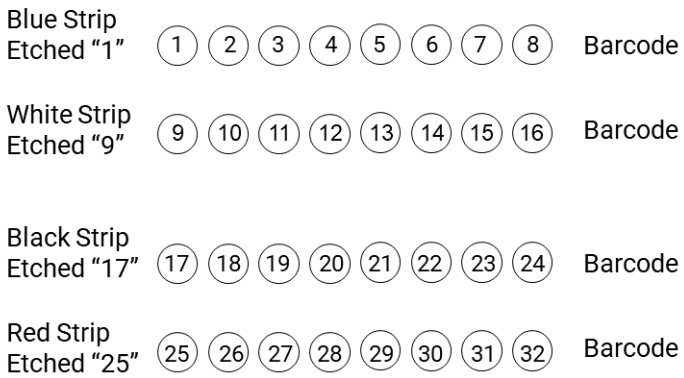

Figure 7 Map of the SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) strip tubes provided with 16 reaction kits

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

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

Index Primer Pairs 17-24 are provided in a black strip, with pair #17 supplied in the well proximal to the numeral *17* etched on the strip's plastic end tab.

Index Primer Pairs 25-32 are provided in a red strip, with pair #25 supplied in the well proximal to the numeral *25* etched on the strip's plastic end tab.

When using the strip tube- supplied index primer pairs in the library preparation protocol, pierce the foil seal of the appropriate well with a pipette tip just before pipetting the solution. If the foil seal for any unused wells is disrupted during use, the unused wells may be re- sealed using the provided fresh foil seal strips. The provided foil strips may also be used to re- seal used wells to prevent index pair cross- contamination during subsequent use.

See Table 71 and Table 72 on page 85 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.

Table 71 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 72 Plate map for SureSelect XT HS2 Index Primer Pairs 97-192, provided in blue plate

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

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 samples contain <50 ng gDNA or total RNA

The SureSelect Cancer CGP Assay requires sample input amounts of 50 ng genomic DNA or 50 ng total RNA for optimal performance including enabling high-confidence discovery of variant alleles down to 5% frequency. The SureSelect XT HS2 reagent system used for library preparation and target enrichment supports use of 10–200 ng DNA or RNA input. Accordingly, libraries can be prepared from as little as 10 ng input, with possible negative impacts on yield and sequencing coverage. If <50 DNA or RNA is recovered for a sample in the run, review the considerations below:

- ✓If additional starting material is available, perform an additional round of gDNA or total RNA isolation for the sample.
- ✓If library preparation is performed using 10-50 ng gDNA or total RNA, increase the pre-capture PCR cycle number by 1 to 2 cycles (add 2 cycles for minimal 10 ng input).
- ✓Library preparation using poor-quality FFPE DNA (DIN<3) or RNA (DV200<50%) at <50 ng input is not recommended, even when using self-optimized conditions.

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 31) is kept at room temperature for 30–45 minutes before use.
- ✓PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the pre-capture PCR cycle number by 1 to 2 cycles. If a high molecular weight peak (>500 bp) is observed in the electropherogram for a sample with low yield, the DNA may be overamplified. Repeat library preparation for the sample, decreasing the pre-capture PCR cycle number by 1 to 3 cycles.
- ✓DNA isolated from FFPE tissue samples may be over-fragmented or have modifications that adversely affect library preparation processes. Use the Agilent NGS FFPE QC Kit to determine the precise quantity of amplifiable DNA in the sample and allow direct normalization of input DNA amount.
- ✓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 over-dried 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 sheared DNA pre-capture library fragment size is larger than expected in electropherograms

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

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

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

✓FFPE DNA or FFPE RNA pre-capture libraries may have a smaller fragment size distribution due to the presence of DNA or RNA fragments in the sample input that are smaller than the targeted post-fragmentation size. Adhere to the DNA quality guidelines provided on page 24 and the RNA quality guidelines provided on page 18.

✓DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP Beads. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color and verify that you are using the bead volume recommended for pre-capture purification on page 36.

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

✓The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to the samples analyzed on page 38. 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 32. 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.

If yield of post-capture libraries is low

✓The probe used for hybridization may have been compromised. Verify the expiration date on the probe vial or Certificate of Analysis. Adhere to the recommended storage and handling conditions. Ensure that the probe hybridization mix is prepared immediately before use, as directed on page 41, and that solutions containing the probe are not held at room temperature for extended periods.

If samples seep from wells during post-hybridization washes

✓Some users experience liquid seepage during post-hybridization wash vortexing or spinning steps, especially when samples are processed in flexible 8-well strip tubes.

- Use of plates or strip tubes with greater rigidity, or use of a rigid tube holder support while vortexing flexible strip tubes, may reduce the incidence of this event.
- For each protocol step that requires removal of cap strips, reseal the wells with a fresh strip of caps. Cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler.

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 44.

If low fraction of reads in targeted region (low percent on target) is observed

✓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 42)
- Samples are maintained at 70°C during washes (see page 42)
- Bead suspensions are mixed thoroughly during washes by pipetting up and down **and** vortexing (see page 43)

✓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 41).

If low strand specificity is observed for SureSelect Cancer CGP RNA Assay samples

✓Low strand-specificity can indicate issues with the RNA library preparation process including the following:

- Contamination of the cDNA library with PCR amplicons or other non-sample derived DNA sources. Adhere to good laboratory hygiene practices, including performance of cDNA synthesis and library preparation steps in an area designated for Pre-PCR work.
- Contamination of the input RNA sample with gDNA. During RNA isolation, adhere to all DNA exclusion and depletion procedures.
- Use of inappropriate cDNA synthesis or PCR amplification reagents. Only use reagents provided with the SureSelect Cancer CGP Assay Kit to prepare RNA libraries for analysis. Do not substitute with reagents from other kits.
- Use of expired or improperly stored cDNA synthesis reagents. In particular, ensure that the First Strand Master Mix is used prior to the kit expiration date and is stored in the amber vial, as provided.

If a high rRNA fraction is reported for SureSelect Cancer CGP RNA Assay samples

✓Ribosomal RNA sequences are not included in the SureSelect Cancer CGP Probe RNA design. Accordingly the majority of any rRNA-derived cDNAs are excluded from the library during the hybridization and capture steps. Ensure that the hybridization and capture steps are performed at the required stringency to minimize the presence of rRNA and other off-target sequences in the library. Adhere to the hybridization and capture stringency precautions described in the Troubleshooting entry for low fraction of reads in targeted region on page 88.

If expected SNV or Indel variants are not detected

✓SNV and Indel detection depends on performing the assay with sufficient coverage and sequencing depth for the frequency of the variant of interest. Detection of variants present at <5% frequency may require analysis using more than 40M reads.

If you want to perform the assay using an unsupported sample type (e.g., ctDNA or needle aspiration sample)

✓Agilent has not validated the SureSelect Cancer CGP Assay using liquid biopsy or needle aspiration samples. Use of these or any other unsupported sample types requires self-optimization of the protocol and validation of results by the user.

✓If self-optimizing the assay for use with liquid biopsy ctDNA samples, Agilent recommends omitting DNA fragmentation from the workflow.

✓If nucleic acids extracted from needle aspiration samples meet the assay input amount (50 ng) and quality requirements, samples may be suitable for the assay with minor optimization of the library preparation and target enrichment workflow segments.

✓Use of any unsupported sample types requires optimization of the NGS and analysis workflow segments. Ensure that the sequencing depth is sufficient for the expected allele frequency associated with the sample type and variant category.

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In This Book

This guide provides instructions for the SureSelect Cancer CGP Assay, a targeted next-generation sequencing (NGS) solution for interrogation of genomic and transcriptomic features of relevance in solid tumors.

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