SureSelect Cancer All-In-One NGS Target Enrichment

Product Overview Guide

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What is All-In-One Target Enrichment?

Agilent's SureSelect Cancer All-In-One is a targeted next-generation sequencing (NGS) solution that enables you to use a single SureSelect assay to interrogate genomic regions of interest for a variety of features including the following:

- SNVs (single nucleotide variations)
- Indels (short insertions and deletions)
- CNVs (copy number variations) including gene amplifications and deletions
- Translocations

The targeted NGS libraries are prepared using the SureSelect$^{XT\ HS}$ or SureSelect^{XT} Low Input Target Enrichment system, using a library of probe oligos that can be designed to capture various types of variants in genes of interest. The resulting enriched NGS libraries are sequenced on the Illumina platform, and then the data are analyzed using Agilent's SureCall software.

The oligos in a SureSelect Cancer All-In-One probe library can be designed to target one or more of the following genomic regions for the specific types of variants detailed in **Table 1**.

Table 1 Variants detected using SureSelect Cancer All-In-One assays

Genomic Region Targeted	Variant Types Detected
Coding exons and exon-intron boundaries	SNVs, Indels
Selected introns	Translocations
Genome-wide and gene-regional CNV backbone regions	CNVs (gene amplifications and deletions)

Custom SureSelect Cancer All-In-One panels of up to 24 Mb are designed using a dedicated workflow in Agilent's SureDesign web application, as detailed on **page 5**. Pre-designed panels designed to capture variants in genes of interest for lung or solid tumor cancer investigations are also available.

Library preparation and target enrichment using a SureSelect Cancer All-In-One panel is done following the standard SureSelect$^{XT\ HS}$ system protocol or SureSelect^{XT} Low Input system protocol, using the additional considerations outlined on **page 6**. Figure1 shows an overview of the SureSelect Cancer All-In-One NGS sample preparation workflow.

Once NGS data is collected, Agilent's SureCall software (v4.1 or later) offers a workflow to analyze the sequencing data from a SureSelect Cancer All-In-One assay. SureCall uses the sequences captured by the variant type-specific probes (see **Table 1**) for the analysis of the corresponding types of variants. The results are accessible using the **Triage View** window and the **Reports** menu. See **page 7** for more information.

SureSelect Cancer All-In-One NGS Solution Workflow

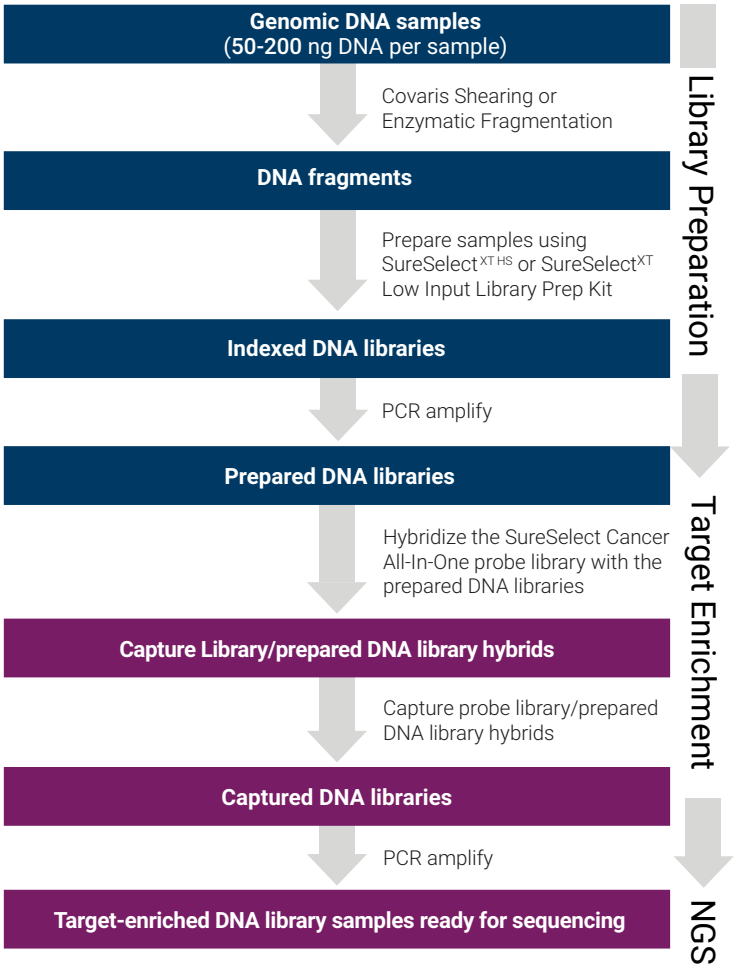

Figure 1. The SureSelect Cancer All-In-One target enrichment workflow.

Kit Part Numbers

The SureSelect Cancer All-In-One system is provided as a bundle of a probe capture library with either a SureSelect$^{XT\ HS}$ reagent kit or a SureSelect^{XT} Low Input reagent kit (see **Table 2**). Custom SureSelect Cancer All-In-One capture libraries are designed using Agilent's SureDesign web application as detailed on **page 5**. Probe designs for the pre-designed SureSelect Cancer All-In-One Lung and Solid Tumor capture libraries may also be accessed using the SureDesign web application.

Table 2 **Reagent Kit + Probe Capture Library Part Numbers**

Probe Capture Library	SureSelect $^{XT\ HS}$ Kits			SureSelect ^{XT} Low Input Kits	
	16 Reactions, with Index Primers 1–16	16 Reactions, with Index Primers 17–32	96 Reactions, with Index Primers 1–32*	96 Reactions, with Index Primers 1–96 (manual/automated)	96 Reactions, with Index Primers 97–192 (manual/automated)
Custom 1–499 kb	G9704A	G9705A	G9706A	G9707A/G9507A	G9708A/G9508A
Custom 0.5 –2.9 Mb	G9704B	G9705B	G9706B	G9707B/G9507B	G9708B/G9508B
Custom 3–5.9 Mb	G9704C	G9705C	G9706C	G9707C/G9507C	G9708C/G9508C
Custom 6–11.9 Mb	G9704D	G9705D	G9706D	G9707D/G9507D	G9708D/G9508D
Custom 12–24 Mb	G9704E	G9705E	G9706E	G9707E/G9507E	G9708E/G9508E
Pre-designed Lung	G9704R	G9705R	G9706R	G9707R/G9507R	G9708R/G9508R
Pre-designed Solid Tumor	G9704S	G9705S	G9706S	G9707S/G9507S	G9708S/G9508S

* Includes 3 single-reaction vials of each index primer 1–32, for a total of 96 library preparations.

Agilent's Human Reference DNAs, listed in Table 3 below, can be used as unmatched reference samples for SureSelect Cancer All-In-One analysis, as detailed on **page 6**.

Table 3 **Optional Agilent Human Reference DNA Products**

Product	Part Number
OneSeq Human Reference DNA, Male	5190-8848
OneSeq Human Reference DNA, Female	5190-8850

Designing Custom SureSelect Cancer All-In-One Panels

Use the wizard-guided *SureSelect All-In-One* design workflow in Agilent's SureDesign web application to design your SureSelect Cancer All-In-One capture panels (see **Figure 2**, below). You can create a custom panel by selecting target genes from a list of pre-defined cancer genes and by entering genes or regions of interest to detect specific types of variants. Special considerations for designs that target CNVs and translocations are discussed below.

For any Cancer All-In-One custom panels that target one or more genes for CNV-detection, a small genome-wide CNV backbone of 100 kb probe size is added in the panel design. In addition, regional backbone probes that are evenly spaced along the transcribed regions of the CNV target genes, plus Agilent-defined padding, are also included. These backbones collectively provide copy number normalization, and single-nucleotide polymorphism (SNP) information for Agilent's SureCall CNV algorithm to estimate the clonal fraction of each CNV call.

Translocations frequently occur in intronic and intergenic regions that are more likely to contain repetitive sequences. Designs containing translocation hotspot regions can have lower capture specificity than exon-focused designs, leading to a lower percentage of reads on-target. In addition, very small designs (<50 kb) may show a suboptimal percentage of reads on-target. Accordingly, if a custom Cancer All-In-One panel design is less than 50 kb, we recommend that you add additional targets or empirically increase the sequencing depth to compensate for the lower percentage of reads on-target. Agilent's Tier 1 custom probe designs can include up to 500 kb of targets.

Your custom panels can be ordered using the **Order** link associated with each panel design on SureDesign.

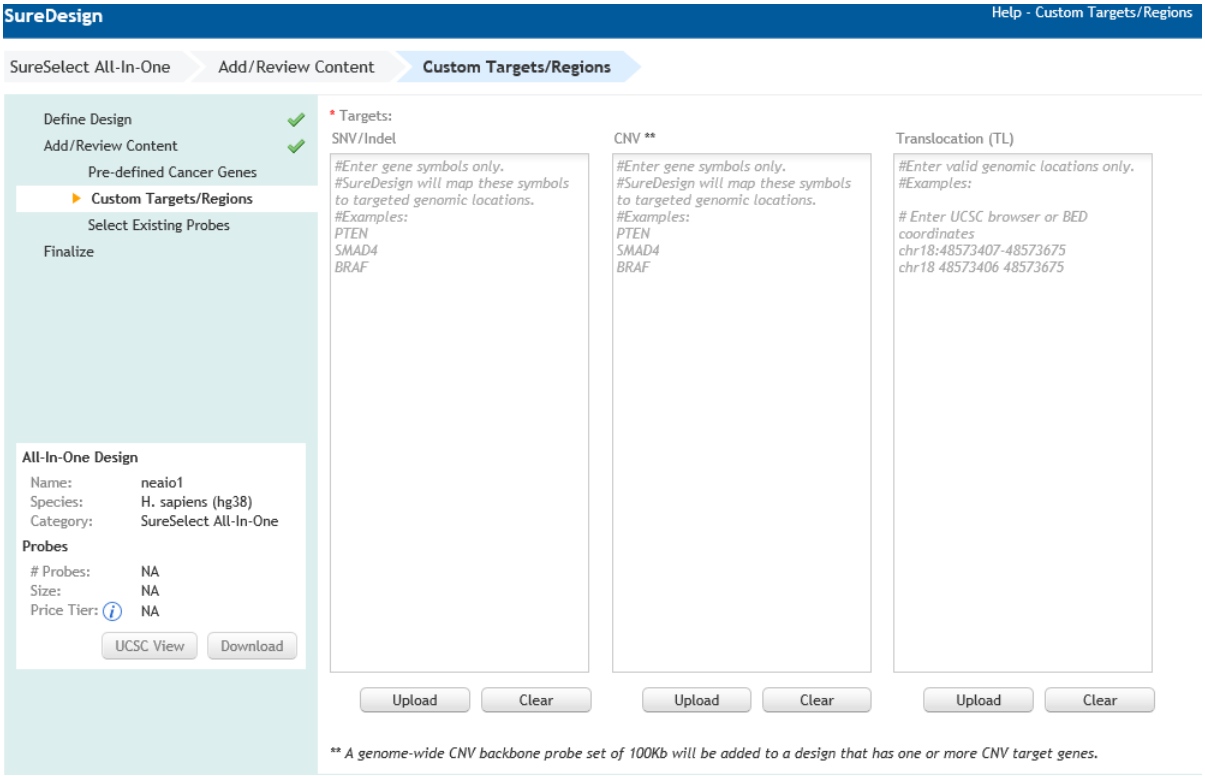

Figure 2. The SureSelect All-In-One custom design interface in SureDesign, used to define genes of interest for specific types of variants.

Protocol Considerations for the SureSelect Cancer All-In-One Assay

Perform library preparation and target enrichment using a SureSelect Cancer All-In-One panel according to the instructions in the SureSelect$^{XT\ HS}$ system protocol or the SureSelect^{XT} Low Input system protocol, using the additional considerations listed below.

Use of a reference sample

In addition to sequence data from the experimental sample, SureSelect Cancer All-In-One system analysis in Agilent's SureCall application requires sequence data from a reference sample (either matched or unmatched) without aberrations in the regions of interest. Reference sample characteristics are especially important for CNV detection. For highest CNV-detection sensitivity, include a matched-normal (non-tumorous tissue) reference sample for tumor-normal paired analysis. Otherwise, include a sex-matched or a mismatched reference sample each time you perform target enrichment on a set of experimental samples. Use 50 ng of the reference DNA as the starting material in the protocol.

NOTE It is possible to use pre-established reference sample data from a sample that was previously captured using the same probe capture library. However, the potential bias due to batch differences may increase the copy number noise and negatively impact the accuracy of CNV-calling.

Agilent's Human Reference DNA products are recommended for use as unmatched reference samples (ordered separately, see Table 3 for information).

If you elect to use your own reference sample instead of an Agilent-supplied reference sample, the key requirement is that the sample is diploid and has no or minimal CNV aberrations. Additionally, you must first validate any non-Agilent reference DNA by analyzing the chosen reference sample against a well-characterized sample with known aberrations to make sure that SureCall is calling CNVs and other aberrations accurately when compared to the chosen reference sample.

Recommended sample types, tumor content and DNA input amount

SureSelect Cancer All-In-One system supports the use of DNA samples isolated from cell lines, fresh-frozen tissues, and FFPE tissue blocks. Agilent has not validated the SureSelect Cancer All-In-One system using DNA isolated from ctDNA or needle aspiration samples. Use of tumor samples with at least 15% tumor cell content is recommended.

The optimal range of input genomic DNA is 50–200 ng for samples to be enriched and analyzed using the SureSelect Cancer All-In-One system. The DNA input range of 10–200 ng listed in the SureSelect$^{XT\ HS}$ or SureSelect^{XT} Low Input Target Enrichment system user manual can be used, with potentially reduced sensitivity for samples processed using <50 ng DNA.

FFPE sample quantitation

For FFPE samples, Agilent recommends sample qualification using the qPCR-based Agilent NGS FFPE QC Kit, and then using the qPCR-determined concentration of amplifiable DNA when preparing input DNA samples. If qPCR methods are unavailable, it is also acceptable to use quantities of input DNA determined using Qubit assays or using Agilent's Genomic DNA ScreenTape assays.

Exclusion of molecular barcodes during analysis with SureCall

The *All-In-One Analysis* workflow in the SureCall software (v4.1) currently does not use molecular barcodes to remove duplicate reads. SureSelect Cancer All-In-One libraries prepared using the SureSelect$^{XT\ HS}$ or SureSelect^{XT} Low Input Target Enrichment system kits and protocols will, however, contain the degenerate molecular barcodes, and it is possible to design custom analysis pipelines that include molecular barcode-based deduplication.

For standard single-indexed samples analyzed with the SureCall *All-In-One Analysis* workflow (see **page 7** for more information), it is not necessary to collect and analyze i5 molecular barcode reads. If your research design includes sample preparation or analysis methods that deviate from this standard workflow, you should determine whether to collect and analyze i5 index reads. Deviations that require i5 index reads include dual-index based demultiplexing using samples prepared with Agilent's Dual Indexing P5 Indexed Adaptors and any custom analysis methods that incorporate i5 molecular barcode reads for single-indexed samples.

Sequencing read length

For translocation detection, Agilent strongly recommends sequencing using at least 2 × 100 bp and preferably 2 × 150 bp paired-end reads. For other applications, follow the read length recommendations in the SureSelect$^{XT\ HS}$ or SureSelect^{XT} Low Input Target Enrichment system user manual.

Sequencing depth

When using 50 ng DNA input, the recommended sequence depth is 4000x or more for 5% variant allele frequency (VAF) and 8000x or more for 1% VAF. Higher sequencing depth may be needed for lower DNA input amounts.

The recommended number of reads for SureSelect Cancer All-In-One Lung and Solid Tumor assays are listed in **Table 4** below.

Table 4 **Read Number Recommendations**

Assay	Target VAF	Recommended Read Number
SureSelect Cancer All-In-One Lung assay	5%	9 million
	1%	18 million
SureSelect Cancer All-In-One Solid Tumor assay	5%	30 million
	1%	60 million

Analyzing the Results in SureCall

Agilent's SureCall software v4.1 has the necessary tools and algorithms for analyzing sequencing data from a SureSelect Cancer All-In-One assay. SureCall can perform the analysis using unaligned FASTQ files or aligned BAM files.

The steps for analyzing SureSelect Cancer All-In-One data in SureCall are summarized in **Figure 3**. The SureCall Help system has specific instructions on each of these steps. Press F1 from any screen in the SureCall software to access the relevant Help topics.

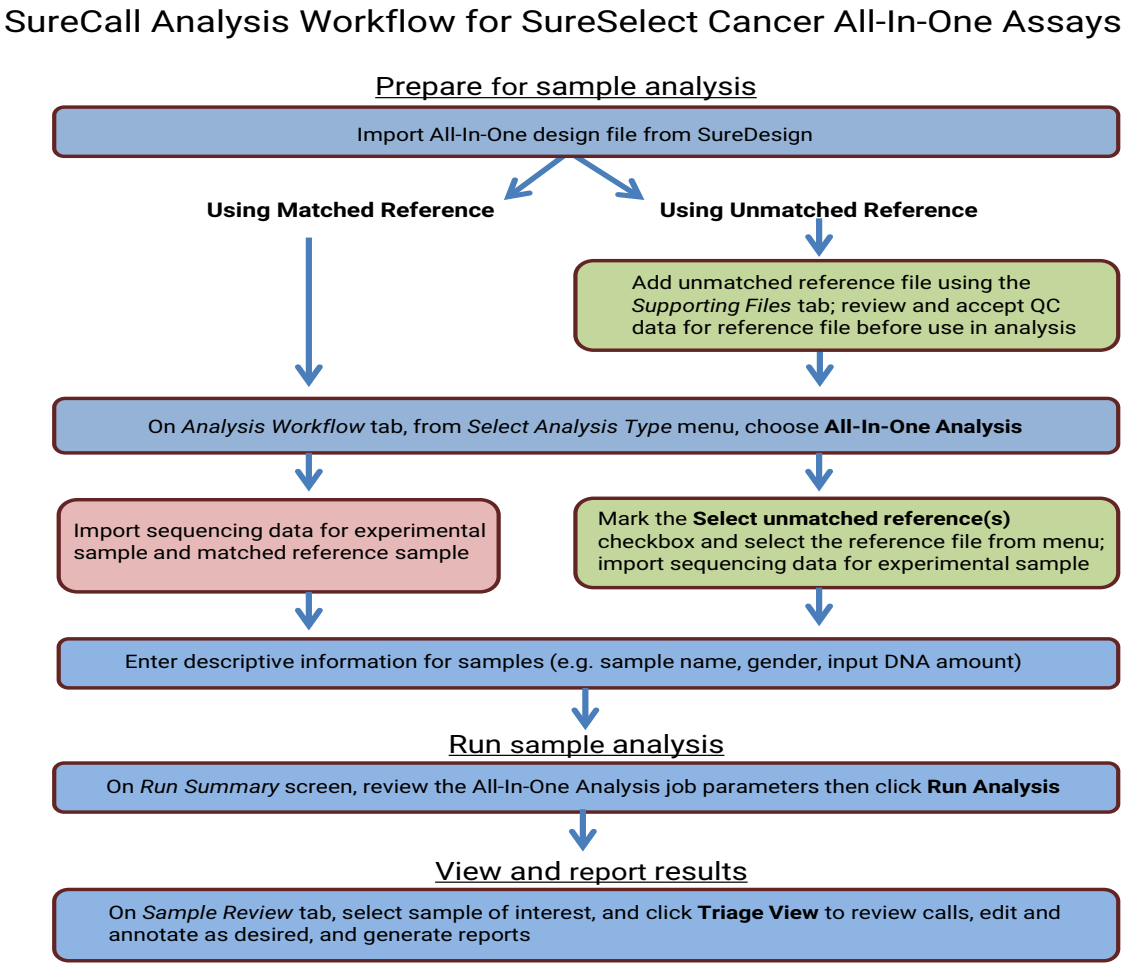

Figure 3. Overview of the All-In-One data analysis workflow in SureCall.

Workflow Guidelines

Sample Characteristics		
Supported Sample Types	**Sample Details**	**Tumor Content**
High-quality DNA samples	Fresh-frozen cells, cell lines and other sample types that typically generate high-quality DNA.	≥15% (measured by Haemotoxylin & Eosin staining)
FFPE DNA samples	Obtained from tissue resection (tissue curls or sections on slide). Recommend minimum of 3 sections of 5 µm.	≥15% (measured by Haemotoxylin & Eosin staining)
Unsupported Sample Types	**Comments**	
ctDNA	SureSelect Cancer All-In-One assays have not been specifically optimized for this sample type, but ctDNA samples may be suitable for SureSelect XT HS or SureSelect XT Low Input library preparation with the modification of omitting DNA fragmentation. For NGS optimization, ensure that the sequencing depth used is appropriate for the expected allele frequency.	
Needle aspiration/needle biopsy	SureSelect Cancer All-In-One assays have not been specifically optimized for this sample type. If DNA extracted from samples meets the recommended DNA input amount (50 ng), samples may be suitable for SureSelect XT HS or SureSelect XT Low Input library preparation. If the QC analysis of the pre-capture library demonstrates the expected quantity and size profile of library DNA, the libraries may be suitable for use in the SureSelect Cancer All-In-One assay target enrichment and NGS analysis workflow steps.	

DNA Extraction and QC			
Supported Sample Type	**Recommended DNA Extraction Kits**	**Optimal DNA QC Method and Entry Criteria**	**Alternative DNA QC Method and Entry Criteria**
High-quality DNA samples	QIAamp DNA Mini Kit (Qiagen)	50–200 ng input DNA, quantified by Qubit BR dsDNA assay (Thermo Fisher Scientific), qualified by AD 260/280	50–200 ng input DNA, quantified by Qubit BR dsDNA assay (Thermo Fisher Scientific) or Agilent TapeStation or Bioanalyzer platform assay, qualified by AD 260/280
FFPE DNA samples	QIAamp DNA FFPE Tissue Kit (Qiagen) or Agencourt FormaPure Kit (Beckman Coulter)	50–200 ng input DNA, qualified by qPCR using Agilent NGS FFPE QC Kit. Recommend ΔΔCq ≤ 5 for SureSelect Cancer All-In-One assays. Quality results dictate method for quantification: for higher-quality samples with ΔΔCq≤ 1, use Qubit-determined concentration, for lower-quality samples with ΔΔCq>1, use qPCR-determined concentration of amplifiable DNA.	50–200 ng input DNA, qualified by Agilent TapeStation Genomic DNA Analysis assay. Recommend DIN ≥ 2 for SureSelect Cancer All-In-One assays. Quantify by Qubit BR dsDNA assay.

Fragmentation		
Supported Sample Type	**Mechanical (Covaris) Shearing Guidelines**	**Enzymatic Fragmentation Guidelines**
High-quality DNA samples	Use two-round shearing (2 × 120 sec) as directed in the SureSelect$^{XT\ HS}$ system protocol or SureSelect^{XT} Low Input system protocol.	Use the DNA fragmentation protocol provided in the SureSelect XT HS and XT Low Input Enzymatic Fragmentation protocol. The protocol requires input DNA samples of 50 to 200 ng in 7 µl volume. The DNA sample volume may be adjusted by dilution with nuclease-free water or by volume reduction using vacuum concentration, DNA spin cups or other suitable DNA concentration methods.
FFPE DNA samples	Use single-round shearing (240 sec) as directed in the SureSelect$^{XT\ HS}$ system protocol or SureSelect^{XT} Low Input system protocol. Use the same DNA shearing conditions for all FFPE samples, including highly degraded samples. Mechanical shearing using the Covaris instrument does not further fragment DNA that is already smaller than the target shear size.	Use the same DNA fragmentation conditions for all samples, including highly degraded FFPE samples, to ensure that the DNA fragment ends are suitable for ligation. See the guidelines provided above for high-quality DNA samples.

Pre-Capture QC, Hybridization, and Post-Capture QC Checkpoints			
Supported Sample Type	**Pre-Capture QC**	**Hybridization Input**	**Post-capture QC**
High-quality DNA samples	Quantify and qualify the pre-capture library DNA using Agilent TapeStation or Bioanalyzer system. Electropherograms show peak of DNA fragment size positioned between 300 to 400 bp, with yield typically ≥ 500 ng DNA.	500–1000 ng prepared library DNA	Quantify and qualify the post-capture libraries using Agilent TapeStation or Bioanalyzer system. Electropherograms show peak of DNA fragment size positioned between 200 to 400 bp. Post-capture library yields are highly variable; proceed with sequencing as long as the yield for each sample meets the requirements of your pooling strategy and NGS platform.
FFPE DNA samples	Quantify and qualify the pre-capture library DNA using Agilent TapeStation or Bioanalyzer system. Electropherograms show peak of DNA fragment size positioned between 200 to 400 bp, with yield typically ≥ 300 ng DNA.	For optimal results hybridize using 500–1000 ng prepared library DNA. In cases where 500 ng of amplified pre-capture library is not available (e.g. for libraries prepared from highly degraded DNA samples), DNA inputs as low as 300 ng may be used, with potential effects on capture performance or NGS metrics.	

Sequencing	
Supported Sample Type	**Library Pooling Guidelines**
High-quality DNA samples	Pool equimolar amounts of post-capture libraries to 2 nM, 4 nM, or 10 nM concentration, depending on your Illumina sequencing sample prep protocol. Dry down the pool using vacuum concentration then resuspend at the required volume. Alternatively, you can adjust the sequencing library pool volume using Solid Phase Reversible Immobilization (SPRI) beads.
FFPE DNA samples	

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In This Book

This guide provides overview information for Agilent's SureSelect Cancer All-In-One system, including considerations for probe capture library design using Agilent's SureDesign application, sample preparation using either SureSelect$^{XT\ HS}$ Reagent Kits or SureSelect^{XT} Low Input Reagent Kits, and analysis using Agilent's SureCall software.

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