SureSelect Max Library Preparation using Sheared DNA

For Illumina Platform NGS

Protocol

Version A0 September 2024

SureSelect platform manufactured with Agilent SurePrint technology.

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

Notices

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Acknowledgment

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In this Guide...

This guide provides an optimized protocol for preparation of Illumina paired-end multiplexed DNA sequencing libraries using the SureSelect Max Library Preparation Module. The SureSelect Max workflow segment supported by this guide includes mechanical Covaris shearing of gDNA samples through library preparation using adaptors with optional duplex molecular barcodes or MBCs. Libraries are PCR-indexed using SureSelect Max UDI primers. The prepared libraries are ready for target enrichment as described in separate guides for later workflow segments. This publication also supports use of the SureSelect Max Library Preparation Module to prepare DNA libraries for whole genome sequencing using a modified protocol.

Content

1 Before You Begin

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

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

Overview of the Workflow

The SureSelect Max system offers flexible workflow options for preparation and target enrichment of DNA or RNA libraries for NGS, with kits for specific workflow segments in a modular format. This publication provides optimized protocols for the DNA library preparation workflow segment using mechanical DNA shearing, summarized in Figure 1. For additional flexibility, libraries can be constructed to include or exclude duplex molecular barcodes (MBCs) by using different library adaptors and can be indexed using 384 unique dual indexing (UDI) primers. For detailed protocols see "DNA Library Preparation with Covaris Shearing Protocol for Target Enrichment" on page 12. Protocols for downstream target enrichment steps are provided in separate publications.

This publication also supports use of the SureSelect Max Library Preparation Module to prepare DNA libraries for whole genome sequencing using a modified protocol detailed in "Appendix: Whole Genome Library Preparation with Covaris Shearing Protocol" on page 26.

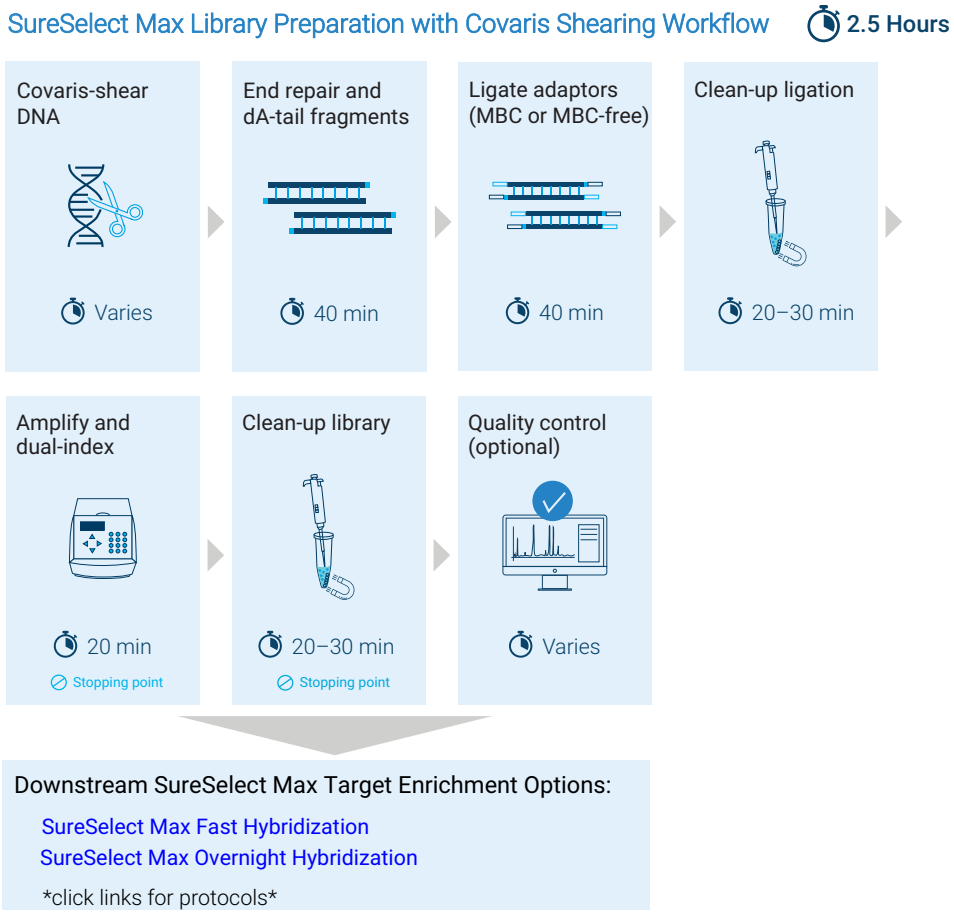

Figure 1 Summary of the SureSelect Max DNA library preparation workflow using mechanical shearing for downstream target enrichment. The estimated time requirements and optional stopping points are provided in this diagram for reference. Estimates are guidelines for 16 reaction runs using 200 ng high-quality input DNA, starting after DNA shearing. Timing for runs using different parameters may vary.

The SureSelect Max system features several improvements over earlier SureSelect platforms:

- Enhanced amplification chemistry and master mixed reagents
- Optional pre-capture QC, with support for capture of undiluted library samples
- Enhanced Fast Hyb chemistry and streamlined capture process
- Faster overall turnaround time with shorter, simplified protocol steps

SureSelect Max reagents are not interchangeable with SureSelect XT HS2 or other SureSelect system reagents.

SureSelect Max Modules Used in the Workflow

This publication provides optimized protocols for the library preparation workflow segment, using mechanical DNA shearing. Agilent's SureSelect reagents required to complete the protocols are summarized in Table 1.

Table 1 SureSelect Max Kits Used in the DNA Library Prep with Mechanical Shearing Workflow

Module Description	16 Reaction Kits	96 Reaction Kits
SureSelect Max DNA Library Prep Kit	G9663A	G9663B
SureSelect Max Adaptors and UDI Primers Kit for ILM (Select One):		
MBC Adaptors and UDI Primers 1-16	G9667A	
MBC Adaptors and UDI Primers 17-32	G9667B	
MBC Adaptors and UDI Primers 1-96		G9668A
MBC Adaptors and UDI Primers 97-192		G9668B
MBC Adaptors and UDI Primers 193-288		G9668C
MBC Adaptors and UDI Primers 289-384		G9668D
MBC-Free Adaptors and UDI Primers 1-16	G9669A	
MBC-Free Adaptors and UDI Primers 17-32	G9669B	
MBC-Free Adaptors and UDI Primers 1-96		G9673A
MBC-Free Adaptors and UDI Primers 97-192		G9673B
MBC-Free Adaptors and UDI Primers 193-288		G9673C
MBC-Free Adaptors and UDI Primers 289-384		G9673D
SureSelect Max Purification Beads*	G9962A (5 mL)	G9962B (30 mL)

* May be substituted with AMPure XP beads (see Table 2).

Additional Materials Used in the Workflow

See Table 2 through Table 4 for additional reagents and equipment used in the workflow.

Table 2 Ordering Information for Additional Reagents and Equipment

Description	Vendor and Part Number	Usage Notes
gDNA isolation and qualification systems	Select from Table 3 on page 10	Select the preparation and qualification systems appropriate for your sample type.
Nucleic acid analysis system	Select from Table 4 on page 10	Prepared library QC is optional prior to downstream target enrichment (see page 7). May also be used for FFPE sample qualification.
Qubit BR dsDNA Assay Kit, 100 assays	Thermo Fisher Scientific p/n Q32850	Use with Thermo Fisher Scientific's Qubit Fluorometer/Assay Tubes (p/n Q33238/Q32856)
Covaris DNA shearing ultrasonicator Covaris microTUBE sample holders	Covaris model E220 Covaris p/n 520045	Alternative Covaris devices may be used after optimization of shearing conditions.
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	—
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, or equivalent	Requires adapter, p/n C1000-ADAPT, for use with strip tubes
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	—
Magnetic separator	Thermo Fisher Scientific p/n 12331D or equivalent	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.
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent	Solvent for gDNA sample preparation and dilution.
Nuclease-free Water	Thermo Fisher Scientific p/n AM9930	Water should not be DEPC-treated.
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276	—
Optional: AMPure XP Kit (5 mL)	Beckman Coulter Genomics p/n A63880	Optional alternative to SureSelect Max Purification Beads (See Table 1)

Table 3 Recommended DNA Sample Isolation and Qualification Systems

Description	Vendor and Part Number	Usage Notes
For preparation of high-quality DNA samples		
High-quality gDNA purification system, for example: QIAamp DNA Mini Kit 50 Samples 250 Samples	 Qiagen p/n 51304 p/n 51306	Recommended reagents for high-quality DNA sample preparation prior to library preparation.
For preparation of FFPE DNA samples		
QIAamp DNA FFPE Tissue Kit, 50 Samples	Qiagen p/n 56404	Recommended reagents for FFPE gDNA sample preparation prior to library preparation.
Deparaffinization Solution	Qiagen p/n 19093	
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. See Table 4 for Agilent TapeStation instrument and accessory ordering information

Table 4 Recommended Nucleic Acid Analysis Systems

Analysis System	Vendor and Part Number Information	Usage Notes
For optional library QC for Target Enrichment workflow		
Agilent 4200/4150 TapeStation Instrument Consumables: 96-well sample plates 96-well plate foil seals 8-well tube strips 8-well tube strip caps D1000 ScreenTape D1000 Reagents	Agilent p/n G2991AA/G2992AA p/n 5042-8502 p/n 5067-5154 p/n 401428 p/n 401425 p/n 5067-5582 p/n 5067-5583	Recommended systems for optional QC of libraries prior to Target Enrichment. (Libraries may also be analyzed using the Agilent 2100 Bioanalyzer instrument and DNA 1000 Kit, p/n 5067-1504.)
Agilent 5200/5300/5400 Fragment Analyzer Instrument Consumables: NGS Fragment Kit (1-6000 bp)	Agilent p/n M5310AA/M5311AA/M5312AA p/n DNF-473-0500	
For required library QC for Whole Genome NGS workflow		
Agilent 4200/4150 TapeStation Instrument Consumables: 96-well sample plates 96-well plate foil seals 8-well tube strips 8-well tube strip caps High Sensitivity D5000 ScreenTape High Sensitivity D5000 Reagents	Agilent p/n G2991AA/G2992AA p/n 5042-8502 p/n 5067-5154 p/n 401428 p/n 401425 p/n 5067-5592 p/n 5067-5593	Required for whole genome library qualification. May be used for whole genome library quantification when libraries amplified using ≥5 PCR cycles (see page 28).
NGS Library Quantification Kit (qPCR-based)	Various suppliers	Required for quantification of whole genome libraries amplified using <5 PCR cycles (see page 28).

Procedural and Safety Notes

- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Use of the following best-practices is recommended 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 Max protocols are highly viscous. Follow the mixing instructions provided in the protocols.
- Avoid introducing bubbles into reaction mixtures during mixing steps. Before adding sample vials to the thermal cycler for incubation or PCR steps, verify the absence of bubbles at the bottom of the sample wells. If present, spin samples briefly to release the bubbles.
- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of caps. Cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler and from other procedural steps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during thermal cycler incubation steps.
- In general, follow Biosafety Level 1 (BSL1) safety rules.
- Possible stopping points, where samples may be stored at 4°C or –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.

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

2 DNA Library Preparation with Covaris Shearing Protocol for Target Enrichment

Use the instructions in this section to prepare DNA libraries for downstream target enrichment.

To prepare DNA libraries for whole genome sequencing, see "Appendix: Whole Genome Library Preparation with Covaris Shearing Protocol" on page 26.

The library preparation protocol is compatible with both high-quality gDNA prepared from fresh or fresh-frozen samples and lower-quality DNA prepared from FFPE samples. The protocol requires 10 ng to 200 ng of input DNA, with adjustments to DNA input amount or quantification method required for some FFPE samples. For optimal sequencing results, use the maximum amount of input DNA available within the recommended range.

Library preparation begins with Covaris-mediated shearing of the DNA samples. The DNA fragments are end-repaired and dA-tailed, and then are ligated to adaptors that either include or exclude duplex molecular barcodes (MBCs). After purification, the library fragments are amplified using unique dual indexing (UDI) primer pairs. After a final purification step, the prepared DNA libraries are ready for later target enrichment and NGS workflow segments. Guidelines are provided at the end of this section for optional QC of the prepared libraries.

For each sample to be sequenced, an individual dual-indexed library is prepared. To process multiple samples together, the protocol includes steps for preparation of reagent mixtures for 8 or 24 samples with overage, followed by distribution to individual DNA samples.

Use of cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA) samples: Agilent has not validated SureSelect Max DNA library preparation using cfDNA or ctDNA samples. If you wish to self-optimize and self-validate library preparation with these sample types, Agilent recommends using the following modifications to the protocols provided in this section:

- Prepare cfDNA or ctDNA samples containing the maximum DNA available in 50 µL 1X Low TE Buffer or nuclease-free water.
- cfDNA or ctDNA is pre-fragmented in blood or similar biological samples. Omit the mechanical shearing steps on page 16 to page 17.
- Otherwise, follow the protocol on page 13 to page 25. Optimization of library amplification cycle number and additional parameters may be required.

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

In preparation for the DNA shearing step on page 16, set up the Covaris instrument according to the manufacturer's instructions. Allow enough time (typically 30–60 minutes) for instrument degassing and water bath chilling before starting the protocol.

Note that the protocols in this section are for library preparation from gDNA samples. To prepare RNA sequencing libraries from cDNA samples using the SureSelect Max Library Preparation Module, see the SureSelect Max RNA Library Preparation Protocol, publication G9664-90000 for important protocol modifications including Adaptor Oligo Mix dilution and amplification conditions suitable for RNA libraries.

Table 5 Reagents thawed before use in protocol

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect Max Library Preparation Module, 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 17
	T4 DNA Ligase (blue cap)	Place on ice just before use, invert to mix.	page 17
	End Repair-A Tailing Buffer (yellow cap or bottle)	Thaw on ice (may require >20 minutes) then keep on ice, vortex to mix.	page 18
	End Repair-A Tailing Enzyme Mix (orange cap)	Place on ice just before use, invert to mix.	page 18
	Amplification Master Mix (red cap or bottle)	Thaw on ice then keep on ice. Mix thoroughly by inversion at least 5X. **Do not vortex.**	page 22
SureSelect Max Adaptors and UDI Primers Kit for ILM, stored at –20°C	**For MBC-tagged libraries**: SureSelect Max MBC Adaptor Oligo Mix, ILM (white cap) OR **For MBC-free libraries**: SureSelect Max MBC-Free Adaptor Oligo Mix (black cap)	Thaw on ice then keep on ice, vortex to mix.	page 19
	SureSelect Max UDI Primers for ILM (select the specific set of indexes to be used in the run): Index Pairs 1-8 (blue strip) Index Pairs 9-16 (white strip) Index Pairs 17-24 (black strip) Index Pairs 25-32 (red strip) Index Pairs 1-96 (orange plate) Index Pairs 97-192 (blue plate) Index Pairs 193-288 (green plate) Index Pairs 289-384 (red plate)	Thaw on ice then keep on ice, vortex to mix.	page 21
+4°C	SureSelect Max Purification Beads OR AMPure XP Beads	Equilibrate at room temperature (RT) for at least 30 minutes before use, vortex to mix. Beads may be retained at RT for both purification steps performed on same day.	page 19 and page 22

Step 1. Prepare and qualify genomic DNA samples

Preparation of high-quality gDNA from fresh biological samples

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

NOTE

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

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

Additional qualification of DNA samples is not required for DNA derived from fresh biological samples. Proceed to "Step 2. Fragment the DNA by Covaris shearing" on page 16.

Preparation and qualification of gDNA from FFPE samples

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

NOTE

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

Store the 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 Quick Guide.

b Consult Table 6 for DIN score-based input DNA input guidelines.

Table 6 DNA input modifications based on DNA Integrity Number (DIN) score

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

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

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.

c 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.

d Use the ΔΔCq score-based guidelines below (summarized in Table 7) to determine the appropriate input DNA quantification method for your sample:

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 7 DNA input guidelines based on ΔΔCq DNA integrity score

ΔΔCq Score	DNA Input Guidelines
ΔΔCq≤1*	10 ng to 200 ng DNA, based on Qubit Assay quantification
ΔΔCq>1	10 ng to 200 ng of amplifiable DNA, based on qPCR quantification

* FFPE samples with ΔΔCq scores ≤1 should be treated like non-FFPE samples for DNA input amount determinations. For samples of this type, make sure to use the DNA concentration determined by the Qubit Assay, instead of the concentration determined by qPCR, to calculate the volume required for 10–200 ng DNA.

Step 2. Fragment the DNA by Covaris shearing

In this step, gDNA samples are sheared using conditions optimized for either high-quality or FFPE DNA in a 50-µL shearing volume to produce DNA fragment sizes suitable for the intended NGS read length. See Table 8 for a summary of shearing duration recommendations. Complete shearing instructions are provided on the following pages.

Table 8 Covaris shearing duration based on NGS read length

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

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

NOTE 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 10–200 ng of gDNA in 50 µL of 1X Low TE Buffer to a final volume of 50 µL. 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 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 9.

Table 9 Shear settings for Covaris E-series instrument (SonoLab software v7 or later)

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

Use the steps below for two-round shearing of high-quality DNA samples only:

- Shear for 120 or 60 seconds (see Table 9)
- Spin the microTUBE for 10 seconds

- Vortex the microTUBE at high speed for 5 seconds
- Spin the microTUBE for 10 seconds
- Shear for additional 120 or 60 seconds
- Spin the microTUBE for 10 seconds
- Vortex the microTUBE at high speed for 5 seconds
- Spin the microTUBE for 10 seconds

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 Spin the microTUBE briefly to collect any residual sample volume and transfer any additional collected liquid to the sample well used in step d.

NOTE

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

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.

Step 3. 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 and step 2 below**.**

1 Vortex the thawed vial of Ligation Buffer for 15 seconds at high speed just before use.

2 Prepare the appropriate volume of ligation master mix by combining the reagents in Table 10.

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 19.

Table 10 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

* 16 reaction Library Preparation Kits contain enough reagents for 2 runs of 8 samples each using the indicated excess volume.

† 96 reaction Library Preparation Kits contain enough reagents for 4 runs of 24 samples each using the indicated excess volume.

Step 4. 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 below.

1 Preprogram a thermal cycler as shown in Table 11. Set the heated lid to 105°C.

Table 11 Thermal cycler program for end repair/dA-tailing (70 µL vol)

Step	Temperature	Time
Step 1	20°C	15 minutes
Step 2	65°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 end repair/dA-tailing master mix by combining the reagents in Table 12.

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 12 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 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 run the program in Table 11.

Step 5. Ligate the adaptor

1 Once the thermal cycler program in Table 11 reaches the 4°C Hold step, transfer the samples to ice. Preprogram the cycler as show in Table 13 with the heated lid off.

Table 13 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 17 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 Max Adaptor Oligo Mix to each sample:

- For **MBC-tagged** libraries–5 µL of SureSelect Max MBC Adaptor Oligo Mix, ILM (white cap)
- For **MBC-free** libraries–5 µL of SureSelect Max MBC-Free Adaptor Oligo Mix (black cap)

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 run the program in Table 13.

NOTE The magnetic purification beads used in the next step must be equilibrated to room temperature for at least 30 minutes before use. Beads can be kept at room temperature through the final pre-capture library purification step on page 22.

Step 6. Purify libraries using magnetic purification beads

CAUTION The bead volume used at this step differs in various SureSelect system protocols. Adhere to the instructions provided here; do not use protocols provided for other SureSelect kits.

Once the thermal cycler program in Table 13 reaches the 4°C hold step, purify the libraries using room-temperature (RT) SureSelect Max Purification Beads or AMPure XP Beads.

Critical purification protocol parameters are summarized for experienced users in Table 14.

Table 14 Magnetic purification bead cleanup parameters after adaptor ligation

Parameter	Value
Volume of RT purification bead suspension added to each sample well	80 µL
Final elution solvent and volume	21 µL nuclease-free water
Amount of eluted sample transferred to fresh well	Approximately 20 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.

2 Mix the room-temperature purification 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.

NOTE In the drying step below, 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.

12 Dry the samples by keeping the unsealed plate or strip tube at room temperature for 2–5 minutes or until the residual ethanol has just evaporated.

13 Elute the library DNA by adding 21 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 2–5 minutes at room temperature. Use of longer incubation times may improve recovery, especially for longer DNA fragments.

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

17 Transfer the cleared supernatant (approximately 20 µL) to a fresh well. Keep on ice.

You can discard the beads at this time.

Step 7. Amplify and index the libraries

1 Determine the appropriate SureSelect Max UDI Primer assignment for each sample. See page 39 for information on the UDI primers used to amplify the DNA libraries in this step.

Use a different UDI number for each sample to be sequenced in the same lane.

NOTE Agilent's SureSelect 8-bp dual indexes use a uniform numbering system across kit formats and between SureSelect Max for ILM and SureSelect XT HS2 platforms. Do not combine samples indexed with the same-numbered dual index pair from different kit formats for multiplex sequencing.

CAUTION The UDI primers 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 15. Set the heated lid to 105°C. Prewarm the instrument before samples are loaded in step 6 on page 22.

Table 15 Library amplification thermal cycler program (50 µL vol)

Segment	Number of Cycles	Temperature	Time
1	1	98°C	45 seconds
2	7 to 13 based on input DNA quality and quantity (see Table 16)	98°C	15 seconds
		60°C	30 seconds
		72°C	30 seconds
3	1	72°C	1 minute
4	1	4°C	Hold

Table 16 Amplification program cycle number recommendations

Quality of Input DNA	Quantity of Input DNA	Cycles
Intact DNA from fresh sample	200 ng	7 cycles
	100 ng	8 cycles
	50 ng	9 cycles
	10 ng	10 cycles
FFPE sample DNA	100 to 200 ng*	11 cycles
	50 ng*	12 cycles
	10 ng*	13 cycles

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

NOTE To avoid cross-contamination, it is recommended to 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 Mix the thawed Amplification Master Mix (red cap or bottle) thoroughly by inversion (do not vortex) then spin briefly.

4 Add 25 µL of the Amplification Master Mix to each sample well containing purified DNA library fragments (20 µL).

5 Add 5 µL of the appropriate SureSelect Max UDI 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 Place the sample plate or strip tube in the thermal cycler and run the program in Table 15.

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.

Step 8. Purify amplified libraries using magnetic purification beads

Once the thermal cycler program in Table 15 reaches the 4°C hold step, purify the libraries using room-temperature SureSelect Max Purification Beads or AMPure XP Beads.

Critical purification protocol parameters are summarized for experienced users in Table 17.

Table 17 Magnetic purification bead cleanup parameters after amplification

Parameter	Value
Volume of RT purification 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 purification beads well until homogeneous and consistent in color.

3 Transfer the amplified library 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.

NOTE In the drying step below, 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.

12 Dry the samples by keeping the unsealed plate or strip tube at room temperature for 2–5 minutes or until the residual ethanol has just evaporated.

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 2–5 minutes at room temperature. Use of longer incubation times may improve recovery, especially for longer DNA fragments.

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

17 Transfer the cleared supernatant (approximately 14 µL) to a fresh well. Keep on ice.

You can discard the beads at this time.

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

Step 9. QC and quantify the libraries (optional)

QC of the prepared libraries is optional, but quantification is required for hybridization workflows using library normalization, including pre-capture pooling. When normalization is not required, the SureSelect Max Target Enrichment post-capture pooling workflows (both Max Fast Hyb and Max Overnight Hyb) support the use of up to 12 µL of unquantified library samples in hybridization.

For workflows that include pre-capture QC, analyze a sample of each library using one of the platforms in Table 18. Follow the instructions in the linked user guide provided for each assay.

Table 18 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 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 19 for fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided in Figure 2 and Figure 3 to illustrate typical results.

Using the 150 bp to 1000 bp region of the electropherogram, determine the average fragment size and the library DNA concentration.

Table 19 Expected library fragment size guidelines

Input type	NGS read length used to select fragmentation duration	Expected average fragment size (150–1000 bp region)
Intact DNA	2 ×100 reads	250 to 450 bp
	2 ×150 reads	380 to 480 bp
FFPE DNA	2 ×100 OR 2 ×150 reads	250 to 350 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 Figure 2. See *Troubleshooting* on page 52 for additional considerations.

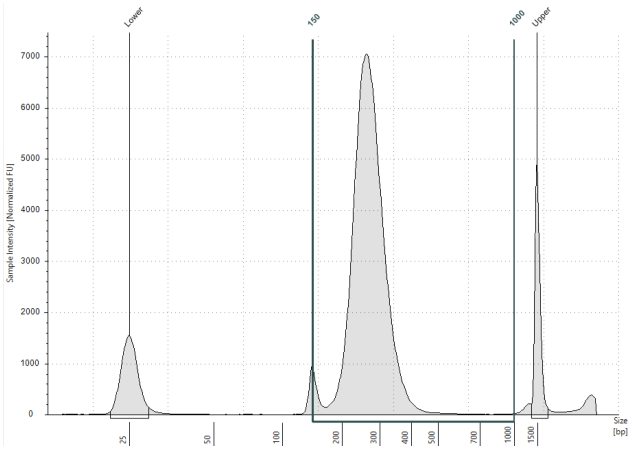

Figure 2 Library prepared from an FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

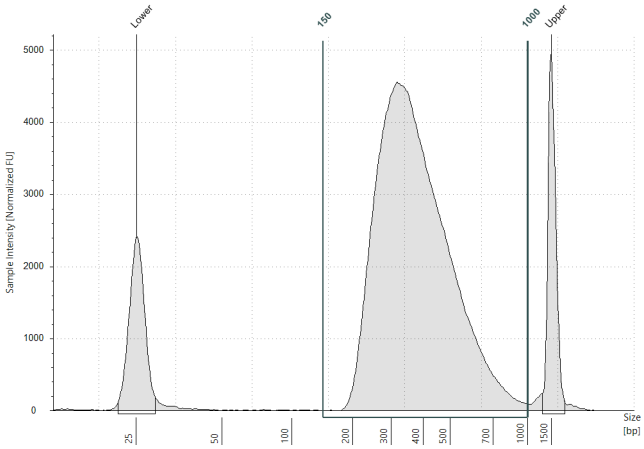

Figure 3 Library prepared from a high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

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

The prepared DNA library fragments are ready for target enrichment using the selected workflow option. Proceed to the appropriate SureSelect Max Target Enrichment Module user guide listed in Table 20 below.

Table 20 Target Enrichment workflow options

Workflow option	Module User Guide link
Max Fast Hybridization (with pre-capture or post-capture pooling)	G9689-90000
Max Overnight Hybridization (with pre-capture or post-capture pooling)	G9690-90000

3
Appendix: Whole Genome Library Preparation with Covaris Shearing Protocol

This appendix provides an optimized protocol for SureSelect Max Library Preparation for Whole Genome Sequencing using Covaris DNA fragmentation, summarized in Figure 4. Guidelines are included in this appendix for NGS using the Illumina platform.

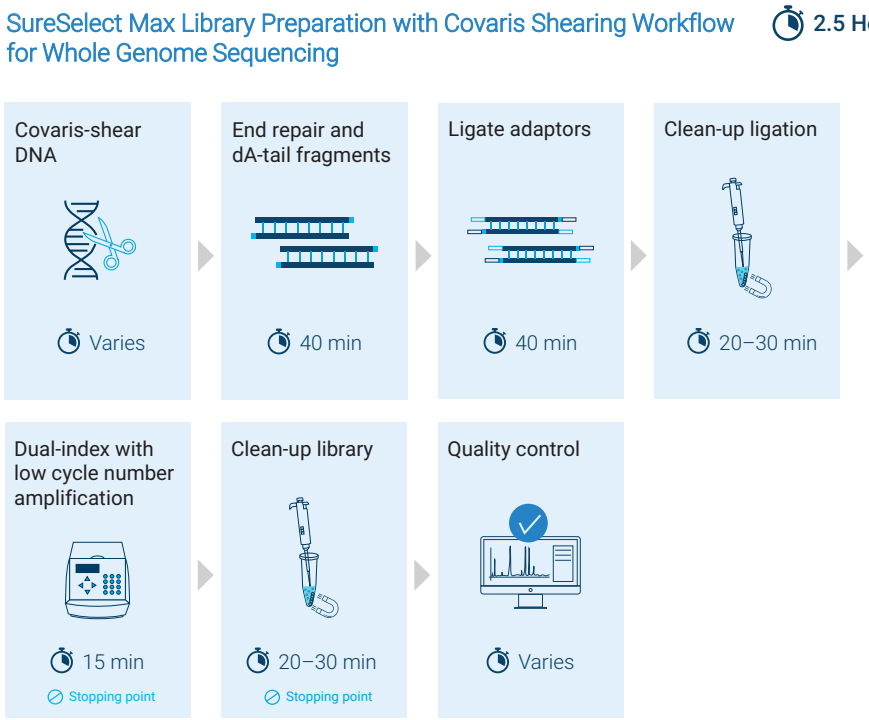

Figure 4 Summary of SureSelect Max DNA library preparation using Covaris shearing for whole genome sequencing. Optional stopping points and estimated time requirements are provided for reference. Estimates are guidelines for 16 reaction runs using 200 ng high-quality input DNA starting after DNA shearing. Timing for runs using different protocol parameters may vary.

Protocol Overview

The whole genome library preparation protocol uses methods and conditions similar to the library preparation protocol for target enrichment provided on page 13 to page 23. This Appendix details the modifications required for whole genome library preparation.

The whole genome library preparation protocol uses the reagents listed in Table 5 on page 13. The SureSelect Max MBC-Free adaptor (black cap) is recommended for use with whole genome sequencing applications. Before you begin, prepare the reagents and the Covaris instrument as directed on page 13.

Use of cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA) samples: Agilent has not validated SureSelect Max whole genome library preparation using cfDNA or ctDNA samples. If you wish to self-optimize and self-validate library preparation with these sample types, Agilent recommends using the following modifications to the protocols provided in this section:

- Prepare cfDNA or ctDNA samples containing the maximum DNA available in 50 µL 1X Low TE Buffer or nuclease-free water.
- cfDNA or ctDNA is pre-fragmented in blood or similar biological samples. Omit "Step 2. Fragment the DNA by Covaris shearing" described below.
- Otherwise, follow the protocols in this section. Optimization of library amplification cycle number and additional parameters may be required.

Step 1. Prepare and qualify genomic DNA samples

Follow the instructions on page 14 to page 15. At the end of this section, you should have samples containing 10–200 ng DNA in 50 µL of Low TE Buffer. If you wish to minimize the cycle number in downstream amplification steps, use the maximum input DNA available in the 10–200 ng range.

Step 2. Fragment the DNA by Covaris shearing

The Whole Genome Library Preparation protocol uses modified shearing conditions to produce longer DNA fragments. Follow the instructions on page 16 to page 17 using the appropriate shearing duration shown in Table 21 below.

At the end of this section, you should have samples containing 10–200 ng sheared DNA in 50 µL of Low TE Buffer.

Table 21 Covaris shearing duration based on NGS read length

NGS read length requirement	Target fragment size	Shearing duration for high-quality DNA samples	Shearing duration for FFPE DNA samples*
2 ×150 reads	400 to 550 bp	2 × 15 seconds	240 seconds
2 ×250 reads	700 to 800 bp	2 × 5 seconds	Not applicable

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

Step 3. Prepare the ligation master mix

Follow the instructions on page 17 (step 1 through step 2), to prepare the ligation master mix for later use in the protocol.

Step 4. Repair and dA-tail the DNA 3' ends

Follow the instructions on page 18 (step 1 through step 5), to dA-tail the 3’ ends of the DNA fragments. At the end of this section, the 70 µL fragmented/A-tailed DNA samples are held in the thermal cycler.

Step 5. Ligate the adaptor

Follow the instructions on page 19 (step 1 through step 4). To prepare MBC-Free whole genome libraries, use the SureSelect Max MBC-Free Adaptor Oligo Mix (black cap) at this step. At the end of this section, the 100 µL adaptor-ligated DNA samples are held in the thermal cycler.

Step 6. Purify libraries using magnetic purification beads

Follow the instructions on page 20 (step 1 through step 17). At the end of this section, the library DNA samples are in approximately 20 µL of nuclease-free water, held on ice.

Step 7. Index and amplify the libraries

The Whole Genome Library Preparation protocol uses a low PCR cycle number to index and amplify the libraries. Follow the instructions provided below for this step.

1 Determine the appropriate SureSelect Max UDI Primer assignment for each sample. Use a different UDI number for each sample to be sequenced in the same lane.

2 Preprogram a thermal cycler as shown in Table 22. Set the heated lid to 105°C. Prewarm the instrument before samples are loaded.

NOTE

Libraries prepared from 50 ng to 200 ng of intact DNA can be indexed using as few as 3 PCR cycles (see Table 23). When indexing using <5 PCR cycles, the final libraries must be quantified for sequencing using qPCR. For libraries amplified with ≥5 cycles, automated electrophoresis may be used for quantification. See page 31 for complete QC guidelines.

Table 22 Library indexing and amplification thermal cycler program (50 µL vol)

Segment	Number of Cycles	Temperature	Time
1	1	98°C	45 seconds
2	3 to 8 based on input DNA quality and quantity (see Table 23)	98°C	15 seconds
		60°C	30 seconds
		72°C	30 seconds
3	1	72°C	1 minute
4	1	4°C	Hold

Table 23 Amplification program cycle number recommendations

Quality of Input DNA	Quantity of Input DNA	Cycles
Intact DNA from fresh sample	50 to 200 ng	3–5 cycles*
	10 ng	6 cycles
FFPE sample DNA	50 to 200 ng†	7 cycles
	10 ng†	8 cycles

* Libraries amplified using <5 cycles require use of qPCR quantification for sequencing (see page 31).

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

3 Mix the thawed Amplification Master Mix (red cap or bottle) thoroughly by inversion (do not vortex) then spin briefly.

4 Add 25 µL of the Amplification Master Mix to each sample well containing purified DNA library fragments (20 µL).

5 Add 5 µL of the appropriate SureSelect Max UDI 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 Place the sample plate or strip tube in the thermal cycler and run the program in Table 22.

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.

Step 8. Purify amplified libraries using magnetic purification beads

Once the amplification program in Table 22 reaches the final 4°C hold step, move the samples to room temperature and purify using room-temperature SureSelect Max Purification Beads or AMPure XP Beads as directed in this section.

The Whole Genome Library Preparation protocol uses different final purification conditions for 2x250 versus 2x150 read length libraries. These differences and other important purification protocol parameters are summarized in Table 24.

Table 24 Magnetic purification bead cleanup parameters after amplification

Step/Parameter	Value	
	For 2×150 read NGS	For 2×250 read NGS
Two-fold dilution of library prior to purification	No	Yes (add 50 µL nuclease-free water per well)
Volume of RT purification bead suspension added to each sample well	50 µL	60 µL
Duration of initial sample/bead binding incubation	10 minutes	10 minutes
Final elution solvent and volume	26 µL Low TE Buffer	26 µL Low TE Buffer
Amount of eluted sample transferred to fresh well	Approximately 25 µL	Approximately 25 µL

Important: For libraries to be sequenced using 2 x 250 read length only, dilute each sample two-fold before starting the procedure below. Dilute by adding 50 µL of nuclease-free water to each 50 µL library sample well.

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

2 Mix room-temperature purification beads well until homogeneous and consistent in color.

3 Add the appropriate volume of bead suspension to each sample well:

   For 2 x 150 NGS libraries: add 50 µL of beads to the 50 µL library samples

   For 2 x 250 NGS libraries: add 60 µL of beads to the 100 µL diluted library samples

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 10 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.

NOTE

In the drying step below, 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.

12 Dry the samples by keeping the unsealed plate or strip tube at room temperature for 2–5 minutes or until the residual ethanol has just evaporated.

13 Elute the library DNA by adding 26 µL of 1X 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 2–5 minutes at room temperature. Use of longer incubation times may improve recovery, especially for longer DNA fragments.

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

17 Transfer the cleared supernatant (approximately 25 µL) to a fresh well. Keep on ice. You can discard the beads at this time.

The prepared DNA libraries are ready for NGS after QC and multiplex pooling.

Stopping Point If you do not continue to QC and library pooling on the same day, seal the wells and store at 4°C overnight or at –20°C for prolonged storage.

Step 9. QC and quantify the libraries

Analyze a sample of each library using the appropriate method(s) summarized in Table 25.

All libraries should be size-qualified using Agilent's TapeStation or Fragment Analyzer system. Libraries indexed using at least 5 PCR cycles (see Table 23 on page 29) can also be quantified using Agilent's automated electrophoresis systems. For libraries prepared using the minimum 3 to 4 amplification cycles, the final libraries must be quantified for sequencing with qPCR.

Table 25 Library analysis guidelines

Purpose	Analysis platform	Assay used at this step	Amount of library sample to analyze
Library fragment size qualification (all libraries) and DNA quantification for libraries amplified using ≥ 5 cycles	Agilent 4200/4150 TapeStation system	Agilent High Sensitivity D5000 ScreenTape Assay	10 ng DNA input libraries: 2 µL of 5-fold dilution
			200 ng DNA input libraries: 2 µL of 50-fold dilution
	Agilent 5200/5300/5400 Fragment Analyzer system	Agilent HS NGS Fragment Kit (1-6000 bp)	10 ng DNA input libraries: 2 µL of 5-fold dilution
			200 ng DNA input libraries: 2 µL of 50-fold dilution
DNA quantification for libraries amplified using <5 cycles	qPCR system	qPCR-based NGS Library Quantification Kit	See manufacturer's protocol

Using the 100 bp to 3500 bp region of the electropherogram, determine the average fragment size and the library DNA concentration. See Table 26 for fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided in Figure 5 and Figure 6 to illustrate typical results.

Table 26 Expected library fragment size guidelines

Input type	NGS read length used to select fragmentation duration	Expected average fragment size (100–3500 bp region)
Intact DNA	2 × 150 reads	510 to 610 bp
	2 ×250 reads	800 to 1000 bp
FFPE DNA	2 ×150 reads	250 to 450 bp

Quantify libraries prepared with <5 amplification cycles using the appropriate qPCR-based NGS Library Quantification Kit. The approximate library concentration from the automated electrophoresis assay can be used to determine the appropriate dilution scheme for qPCR quantification.

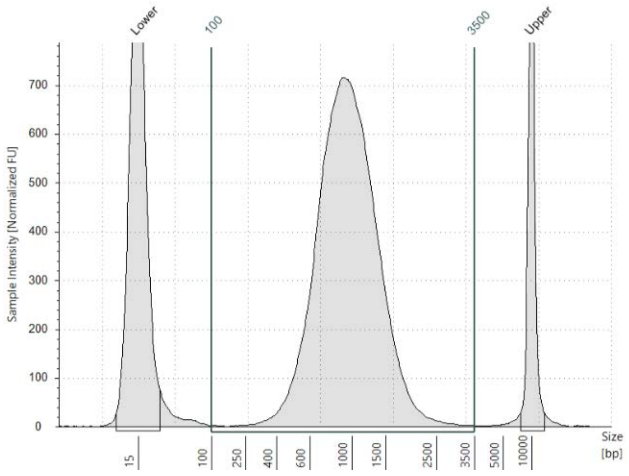

Figure 5 Library prepared from a high-quality gDNA sample using shearing conditions for 2 x 250 bp NGS analyzed using a High Sensitivity D5000 ScreenTape assay.

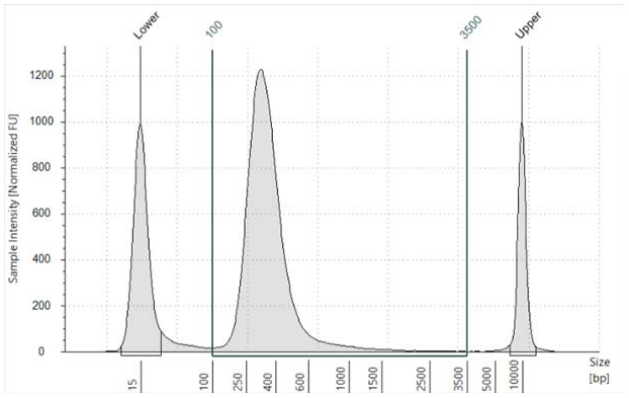

Figure 6 Library prepared from an FFPE gDNA sample using shearing conditions for 2 x 150 bp NGS analyzed using a High Sensitivity D5000 ScreenTape assay.

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.

NGS Guidelines

Library pooling guidelines for multiplex NGS

The SureSelect Max whole genome libraries are ready for pooling for multiplex NGS.

NOTE

SureSelect Max UDI strip and plate layouts are designed to provide the proper color balance for Illumina's two-channel and four-channel systems. A minimum plexity of four is recommended to ensure that library pools are color balanced. Pools containing any four consecutive SureSelect Max UDIs meet Illumina's guidance for optimal color balance and sequencing performance. Consult Illumina's guidelines for additional color balance and pooling strategy information including two-plex or three-plex pooling considerations.

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 for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample.

Combine the library samples such that each indexed library is present in equimolar amounts in the NGS 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 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 NGS pool.

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

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

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

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

is the number of indexes, and

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

Table 27 shows an example of the amount of 4 indexed samples (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM DNA.

Table 27 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

Store the library pool under the conditions specified by your sequencing provider. Typically, libraries should be stored at –20°C for short-term storage.

Sequencing setup and run guidelines

The pooled libraries are 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 (see page 39 for a library structure diagram). Motifs include unique 8-bp P5 and P7 indexes suitable for Illumina sequencing platforms. See page 39 for SureSelect Max UDI information.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. Table 28 provides guidelines for use of several Illumina instrument and chemistry combinations suitable for processing the SureSelect Max DNA whole genome NGS libraries quantified using an Agilent automated electrophoresis system. Consult Illumina's documentation for sequencing setup guidelines for qPCR-quantified libraries and for runs using other Illumina instruments.

Table 28 Illumina Kit Configuration Selection Guidelines

Instrument	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
MiSeq	All Runs	2 × 150 bp or 2 × 250 bp	300 Cycle Kit or 500 Cycle Kit	v2	9–10 pM
			600 Cycle Kit	v3	12–16 pM
NextSeq 1000/2000	All Runs	2 × 150 bp or 2 × 250 bp	300 Cycle Kit or 600 Cycle Kit	Standard SBS	650–1000 pM
			300 Cycle Kit	XLEAP-SBS	650–1000 pM
NovaSeq 6000	Standard Workflow Runs	2 × 150 bp or 2 × 250 bp	300 Cycle Kit or 500 Cycle Kit	v1.5	300–600 pM
	Xp Workflow Runs	2 × 150 bp or 2 × 250 bp	300 Cycle Kit or 500 Cycle Kit	v1.5	200–400 pM
iSeq 100	All Runs	2 × 150 bp	300 Cycle Kit	v2	50–150 pM
NextSeq 500/550	All Runs	2 × 150 bp	300 Cycle Kit	v2.5	1.2–1.5 pM
NovaSeq X	All runs	2 × 150 bp	300 Cycle Kit	v1	90–180 pM

Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in Table 28 or provided by Illumina. Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

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 29 showing example settings for 2x150 bp sequencing.

Table 29 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 information, see page 39.
- No custom primers are used for SureSelect Max library sequencing. Leave all *Custom Primers* options for *Read 1*, *Read 2*, *Index 1* and *Index 2* primers cleared/deselected during run setup.
- 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. If you need assistance with SureSelect Max 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.

Analysis pipeline guidelines

Guidelines are provided below for typical NGS read processing and analysis pipeline steps for SureSelect Max MBC-free whole genome DNA libraries. Your NGS analysis pipeline may vary.

- Demultiplex using Illumina's bcl2fastq, BCL Convert or DRAGEN software to generate paired end reads based on the dual indexes and remove sequences with incorrectly paired P5 and P7 indexes. Sequencing adaptors can also be trimmed from MBC-free reads in this step by turning on the adaptor trimming tools in Illumina's demultiplexing software.
- Align the trimmed reads using a suitable tool such as BWA-MEM.

The resulting BAM files are ready for downstream analysis including variant discovery.

NOTE

If your SureSelect Max whole genome libraries were prepared using MBC Adaptors, reads should be processed using Agilent's AGeNT software to ensure proper adaptor trimming and MBC-based consensus read generation. AGeNT is a Java-based toolkit for library read processing steps, designed for users with bioinformatics expertise to enable building internal analysis pipelines. To download this toolkit, visit the AGeNT page at www.agilent.com. See the AGeNT Best Practices document for instructions. Instructions provided for the SureSelect XT HS2 platform are also applicable to the SureSelect Max platform.

4
Reference

This section contains reference information, including Reagent Kit contents, index sequences, troubleshooting information and quick- reference protocols for experienced users.

Reagent Kit Contents

SureSelect Max DNA Library Preparation with Covaris shearing uses the kits listed in Table 30. Detailed contents of the multi-part component kits are shown in Table 31 through Table 33.

Table 30 Kits for SureSelect Max DNA Library Preparation using Covaris Shearing

Purchased Kit	Included Component Kits	Component Kit Part Number		Storage Condition
		16 Reactions	96 Reactions	
SureSelect Max DNA Library Preparation Kit	SureSelect Max Library Preparation Module	5280-0065	5280-0066	–20°C
SureSelect Max Adaptors and UDI Primers Kit for ILM	SureSelect Max MBC Adaptor Oligo Mix for ILM **OR** SureSelect Max MBC-Free Adaptor Oligo Mix for ILM	5282-0124 **OR** 5282-0126	5282-0125 **OR** 5282-0127	–20°C
	SureSelect Max UDI Primers for ILM	5282-0138 (Index 1-16) 5282-0119 (Index 17-32)	5282-0120 (Index 1-96) 5282-0121 (Index 97-192) 5282-0122 (Index 193-288) 5282-0123 (Index 289-384)	–20°C
SureSelect Max Purification Beads		5282-0225	5282-0226	+4°C

Component Kit Details

Table 31 SureSelect Max Library Preparation Module content

Kit Component	16 Reaction Kit (p/n 5280-0065)	96 Reaction Kit (p/n 5280-0066)
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
Amplification Master Mix	tube with red cap	bottle

Table 32 SureSelect Max Adaptor Oligo Mix for ILM options

Kit Component	16 Reaction Kits	96 Reaction Kits
SureSelect MBC Adaptor Oligo Mix for ILM	tube with white cap	tube with white cap
SureSelect MBC-Free Adaptor Oligo Mix for ILM	tube with black cap	tube with black cap

Table 33 SureSelect Max UDI Primers for ILM options

Kit Component	16 Reaction Kit Format	96 Reaction Kit Format
SureSelect Max UDI Primers for ILM*	Blue 8-well strip tube (index pairs 1-8), AND White 8-well strip tube (index pairs 9-16) **OR** 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), OR Blue 96-well plate (index pairs 97–192), OR Green 96-well plate (index pairs 193–288), OR Red 96-well plate (index pairs 289–384)

* See page 40 through page 42 for index strip and plate position maps; see page 43 through page 50 for index pair sequence information.

SureSelect Max Library Composition

The final SureSelect Max library fragments prepared using Agilent's Illumina-compatible kit modules are shown in Figure 7. Each fragment in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using Illumina sequencers. The libraries are ready for sequencing using standard Illumina paired-end primers and chemistry.

Each library DNA fragment contains a unique 8-bp P5 and P7 index suitable for Illumina sequencing platforms. Additional SureSelect Max UDI Primers Information is provided below.

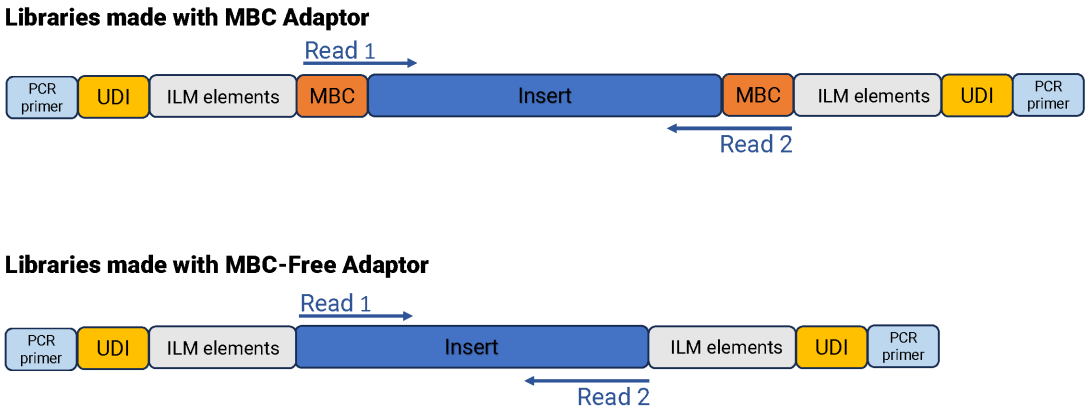

Figure 7 Content of SureSelect Max for ILM sequencing libraries. Each fragment contains one target insert (dark blue) surrounded by the following elements: Illumina paired-end sequencing elements (gray), unique dual indexes (gold), library PCR primers (light blue) and optional molecular barcode (MBC) sequences. MBCs include a 3-bp degenerate barcode plus dark bases.

SureSelect Max UDI Primers Information

The SureSelect Max unique dual indexing (UDI) Primers are provided in pre-combined pairs of indexes. 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 8) or of 96-well plates (96 reaction kits; see page 41 to page 42 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 38 on page 43 through Table 45 on page 50. Index sequences can also be obtained by downloading the SureSelect Max 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 38 through Table 45 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 both forward and reverse complement orientations. Refer to Illumina support documentation and resources to determine the correct P5 index orientation for your application.

Index Primer Pair Strip Tube and Plate Maps

SureSelect Max UDI Primers 1-16 and 17-32 (provided with 16 reaction kits) are supplied in sets of two 8-well strip tubes as detailed below.

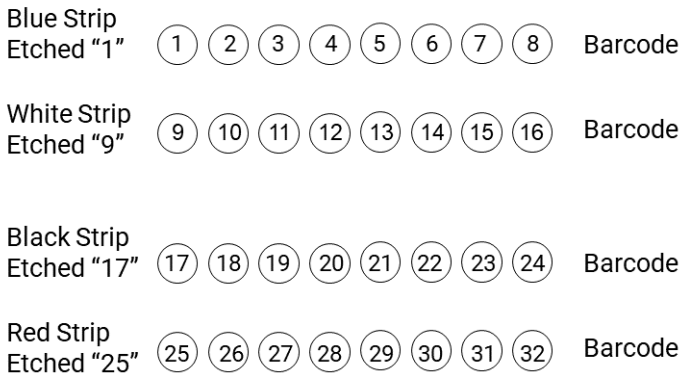

Figure 8 Map of the SureSelect Max UDI Primers for ILM strip tubes in 16 reaction kits

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

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

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

The red strip contains Index Primer Pairs 25-32, 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.

Plate positions of the SureSelect Max UDI Primers for ILM provided with 96 reaction kits are shown in Table 34 through Table 37.

CAUTION The SureSelect Max UDI Primers 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 34 Plate map for SureSelect Max UDI Primers 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 35 Plate map for SureSelect Max UDI Primers 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

Table 36 Plate map for SureSelect **Max UDI Primers** 193-288, provided in green plate

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

Table 37 Plate map for SureSelect **Max UDI Primers** 289-384, provided in red plate

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

SureSelect Max Index Sequences

Table 38 SureSelect Max 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 39 SureSelect Max 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 40 SureSelect Max 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 41 SureSelect Max 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

Table 42 SureSelect Max Index Primer Pairs 193–240, provided in green 96-well plate

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

Table 43 SureSelect Max Index Primer Pairs 241–288, provided in green 96-well plate

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

Table 44 SureSelect Max Index Primer Pairs 289–336, provided in red 96-well plate

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

Table 45 SureSelect Max Index Primer Pairs 337–384, provided in red 96-well plate

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

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 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 yield of libraries is low

✓Use only 1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA) where specified in the protocol for sample preparation and dilution steps. Do not substitute with water or other solvents.

✓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.

✓PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the 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 over-amplified. Repeat library preparation for the sample, decreasing the 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.

✓Yield from the magnetic bead purification step may be suboptimal. Consider the factors below:

- Adhere to all of the bead and reagent handling steps in the protocol. In particular, make sure to equilibrate beads at room temperature for at least 30 minutes before use, and use freshly-prepared 70% ethanol (prepared on day of use) for each purification procedure.
- Ensure that the magnetic beads are not over-dried just prior to sample elution (see step 12 on page 20 and page 23). Monitor the bead pellets frequently while drying and conclude the drying step immediately after the residual ethanol has evaporated.
- Increasing the elution incubation time (up to 10 minutes) may improve recovery, especially for longer DNA fragments.

If library fragment size is different than expected or required in electropherograms

✓FFPE DNA pre-capture libraries may have a smaller fragment size distribution due to the presence of DNA fragments in the sample input that are smaller than the targeted post-fragmentation size. Adhere to the DNA quality guidelines provided on page 15.

✓DNA fragment size selection during purification depends upon using the correct ratio of sample to magnetic purification beads. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color. Verify that you are using the recommended bead volume at each stage of the protocol.

✓For the whole genome sequencing workflow using conditions for 2x150 NGS, the average library fragment size can be increased by adjusting the ratio of bead suspension to DNA sample volumes in the final library purification procedure on page 29. The standard protocol uses beads at 1X the library DNA sample volume. To increase the average fragment size in the library, decrease the bead volume ratio to

the range of 0.6X to 0.7X (with 30 µL or 35 µL of purification bead suspension added to each 50 µL sample). Note that this protocol modification will also decrease the overall library yield.

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 25. 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 19. 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.

Quick Reference Protocol: Library Prep for Target Enrichment

An abbreviated protocol summary is provided below for experienced users. Use the complete protocol on page 13 to page 25 until you are familiar with all of the protocol details.

Step	Summary of Conditions
Prepare and qualify DNA samples	Prepare 10–200 ng gDNA samples in 50 µL 1X Low TE Buffer. For FFPE DNA, qualify integrity and adjust input amount as directed on page 15.
Shear DNA samples	Shear 50 µL DNA samples using Covaris (Table 46)> transfer to plate or strip wells> keep on ice
Prepare Ligation master mix	**Per 8 reactions:** 207 µL Ligation Buffer + 18 µL T4 DNA Ligase **Per 24 reactions:** 598 µL Ligation Buffer + 52 µL T4 DNA Ligase Prepare at room temperature (RT)> mix> spin> keep at RT 30–45 min before use.
Prepare End-Repair/dA-Tailing master mix	**Per 8 reactions:** 144 µL End Repair-A Tailing Buffer + 36 µL End Repair-A Tailing Enzyme Mix **Per 24 reactions:** 416 µL End Repair-A Tailing Buffer + 104 µL End Repair-A Tailing Enzyme Mix Prepare on ice> mix> spin> keep on ice.
End-Repair and dA-Tail the DNA fragments	50 µL sheared DNA + 20 µL End Repair/dA-Tailing master mix Mix> spin> incubate in thermal cycler: 15 min @ 20°C, 15 min @ 65°C, Hold @ 4°C.
Ligate adaptor	70 µL DNA sample + 25 µL Ligation master mix> mix> spin. Add 5 µL SureSelect Max Adaptor Oligo Mix (MBC or MBC-free)> mix> spin. Incubate in thermal cycler: 30 min @ 20°C, Hold @ 4°C.
Purify DNA	100 µL DNA sample + 80 µL purification bead suspension> mix> incubate 5 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute DNA in 21 µL nuclease-free H_2O> mix> incubate 2-5 minutes> collect beads> transfer 20 µL supernatant to fresh well.
Index and amplify library	20 µL DNA sample + 25 µL Amplification Master Mix + 5 µL SureSelect Max UDI Primers for ILM Vortex> spin> amplify in thermal cycler using program in Table 47.
Purify amplified library DNA	50 µL amplified DNA + 50 µL purification bead suspension> mix> incubate 5 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute DNA in 15 µL nuclease-free H_2O> mix> incubate 2-5 minutes> collect beads> transfer 14 µL supernatant to fresh well.
Quantify and qualify DNA	Optional: Analyze quantity and quality using TapeStation or Fragment Analyzer System

Table 46 Shearing conditions for target enrichment libraries

Input DNA	NGS Read Length	Shearing Time
High quality DNA	2 ×100 reads	2 × 120 seconds
	2 ×150 reads	2 × 60 seconds
FFPE-derived DNA	2 ×100 or 2 ×150 reads	240 seconds

Table 47 Library amplification thermal cycler program for target enrichment libraries (50 µL vol; heated lid at 105°C)

Segment	Number of Cycles			Temperature	Time
1	1			98°C	45 seconds
2	Intact DNA input 200 ng: 7 cycles 100 ng: 8 cycles 50 ng: 9 cycles 10 ng: 10 cycles	**OR**	FFPE DNA input 100–200 ng: 11 cycles 50 ng: 12 cycles 10 ng: 13 cycles	98°C 60°C 72°C	15 seconds 30 seconds 30 seconds
3	1			72°C	1 minute
4	1			4°C	Hold

Quick Reference Protocol: Library Prep for Whole Genome NGS

An abbreviated protocol summary is provided below for experienced users. Use the complete protocol on page 27 to page 31 until you are familiar with all of the protocol details.

Step	Summary of Conditions
Prepare and qualify DNA samples	Prepare 10–200 ng gDNA samples in 50 µL 1X Low TE Buffer. For FFPE DNA, qualify integrity and adjust input amount as directed on page 15.
Shear DNA samples	Shear 50 µL DNA samples using Covaris (Table 48) > transfer to plate or strip wells> keep on ice.
Prepare Ligation master mix	**Per 8 reactions:** 207 µL Ligation Buffer + 18 µL T4 DNA Ligase **Per 24 reactions:** 598 µL Ligation Buffer + 52 µL T4 DNA Ligase Prepare at room temperature (RT)> mix> spin> keep at RT 30–45 min before use.
Prepare End-Repair/dA-Tailing master mix	**Per 8 reactions:** 144 µL End Repair-A Tailing Buffer + 36 µL End Repair-A Tailing Enzyme Mix **Per 24 reactions:** 416 µL End Repair-A Tailing Buffer + 104 µL End Repair-A Tailing Enzyme Mix Prepare on ice> mix> spin> keep on ice.
End-Repair and dA-Tail the DNA fragments	50 µL sheared DNA + 20 µL End Repair/dA-Tailing master mix Mix> spin> incubate in thermal cycler: 15 min @ 20°C, 15 min @ 65°C, Hold @ 4°C
Ligate adaptor	70 µL DNA sample + 25 µL Ligation master mix> mix> spin. Add 5 µL SureSelect Max MBC-free Adaptor Oligo Mix> mix> spin. Incubate in thermal cycler: 30 min @ 20°C, Hold @ 4°C
Purify DNA	100 µL DNA sample + 80 µL purification bead suspension> mix> incubate 5 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute DNA in 21 µL nuclease-free H_2O> mix> incubate 2-5 minutes> collect beads> transfer 20 µL supernatant to fresh well.
Index and amplify library	20 µL DNA sample + 25 µL Amplification Master Mix + 5 µL SureSelect Max UDI Primers for ILM Vortex> spin> amplify in thermal cycler using program in Table 49
For 2x250 only, dilute 2X	For libraries to be sequenced using 2x250 reads, add 50 µL of nuclease-free H_2O per sample well.
Purify amplified library DNA	**For 2x150 samples:** 50 µL amplified DNA + 50 µL purification bead suspension **For 2x250 samples:** 100 µL amplified DNA dilution + 60 µL purification bead suspension Mix> incubate 10 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute DNA in 26 µL Low TE Buffer> mix> incubate 2-5 minutes> collect beads> transfer 25 µL supernatant to fresh well.
Quantify and qualify DNA	QC by TapeStation or Fragment Analyzer. Quantify library DNA for NGS using qPCR for 3- or 4-cycle amplified libraries or using TapeStation or Fragment Analyzer for libraries amplified with ≥5 cycles.

Table 48 Shearing conditions for whole genome libraries

Input DNA	NGS Read Length	Shearing Time
High quality DNA	2×150 reads	2 × 15 seconds
	2×250 reads	2 × 5 seconds
FFPE-derived DNA	2×150 reads	240 seconds

Table 49 Library amplification thermal cycler program for whole genome libraries (50 µL vol; heated lid at 105°C)

Segment	Number of Cycles	Temperature	Time
1	1	98°C	45 seconds
2	50–200 ng Intact DNA input libraries: 3–5 cycles 10 ng Intact DNA input libraries: 6 cycles 50–200 ng FFPE DNA input libraries: 7 cycles 10 ng FFPE DNA input libraries: 8 cycles	98°C	15 seconds
		60°C	30 seconds
		72°C	30 seconds
3	1	72°C	1 minute
4	1	4°C	Hold

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In This Book

This guide provides instructions for SureSelect Max DNA Library Preparation using mechanically-sheared DNA for Illumina sequencing.

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