

SureSelect Max PCR-Free Whole Genome Library Preparation using Sheared DNA

For Illumina Platform NGS

Protocol

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In this Guide...

This guide provides an optimized protocol for PCR-free whole genome DNA library preparation from mechanically-sheared DNA, using the SureSelect Max system with full-length SureSelect Max UDI adaptors. Prepared libraries are ready for multiplexed whole-genome sequencing (WGS) on the Illumina platform.

1 Before You Begin

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

2 PCR-Free DNA Library Preparation using Sheared DNA Protocol

This section describes the steps for Covaris shearing of gDNA samples and PCR-free library construction using full-length SureSelect Max UDI Adaptors for WGS. The prepared libraries are quantified by qPCR and qualified by automated electrophoresis prior to pooling for multiplex whole genome NGS.

3 Appendix: NGS Guidelines

This section provides guidelines for downstream NGS on the Illumina platform.

4 Reference

This section contains reference information, including reagent kit contents, adaptor UDI sequences, and troubleshooting information.

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1

Before You Begin

Overview of the Workflow [6](#)
SureSelect Max Modules Used in the Workflow [7](#)
Additional Materials Used in the Workflow [7](#)
Procedural and Safety Notes [9](#)

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

Overview of the Workflow

The SureSelect Max system offers flexible workflow options for NGS library preparation in a modular format. This publication provides optimized protocols for PCR-free whole genome DNA library preparation, using Covaris-sheared DNA. The workflow is summarized in [Figure 1](#). After DNA samples are sheared, libraries are constructed by DNA end-repair and dA-tailing in a single reaction, followed by ligation of full-length adaptors with unique dual indexes (UDIs). The libraries are then purified, qualified and quantified to pool for multiplex NGS on an Illumina platform. For detailed protocols see ["PCR-Free DNA Library Preparation using Sheared DNA Protocol"](#) on page 10.

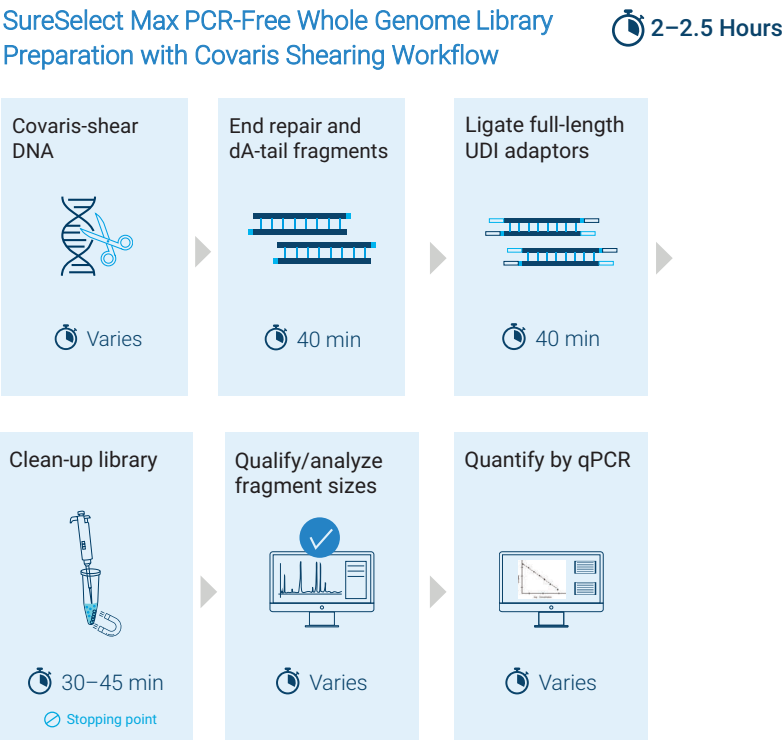


Figure 1 Summary of the SureSelect Max PCR-free whole genome DNA library preparation with sheared DNA workflow. The estimated time requirements and optional stopping points are provided in this diagram for reference. Estimates are guidelines for 16 reaction runs using high-quality input DNA. Timing for runs using different parameters may vary.

SureSelect Max Modules Used in the Workflow

This publication provides optimized protocols for PCR-free whole genome DNA library preparation, using Covaris-sheared DNA samples. Agilent's SureSelect Max reagents required to complete the protocols are summarized in [Table 1](#).

Table 1 Reagent Kits for SureSelect Max PCR-free WGS Library Prep with Covaris Shearing

Module Description	16 Reaction Kits*	96 Reaction Kits†
SureSelect Max Library Prep Kit, PCR-free	G9613A	G9613B
SureSelect Max UDI Adaptors for WGS, PCR-free (Select One): with ILM UDI Adaptors 1-16 with ILM UDI Adaptors 1-96	G9620A	G9620B
SureSelect Max Purification Beads	G9962A (5 mL)	G9962B (30 mL)

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

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

NOTE

AMPure XP Beads can replace SureSelect Max Purification Beads in the protocols provided in this user guide (see [Table 3](#) for ordering information).

Both types of magnetic purification beads have similar overall performance. The bead types are not, however, identical in composition and the average fragment length and yield results may differ slightly. Use a single bead source in validated protocols.

Additional Materials Used in the Workflow

The protocol requires 100–500 ng DNA input. See [Table 2](#) for recommended systems for the preparation and qualification of DNA samples based on sample type. See [Table 3](#) for additional reagents and equipment used in other workflow segments.

Table 2 Recommended DNA Sample Isolation and Qualification Systems

Description	Vendor and Part Number	Usage Notes
For preparation of DNA samples from fresh or fresh-frozen sources		
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	Agilent p/n G9700A p/n G9700B	Recommended systems for FFPE gDNA qualification prior to library preparation. See Table 3 for Agilent TapeStation instrument and accessory ordering information.
OR TapeStation Genomic DNA Analysis Consumables: Agilent Genomic DNA ScreenTape Genomic DNA Reagents	 p/n 5067-5365 p/n 5067-5366	

Table 3 Ordering Information for Additional Reagents and Equipment

Description	Vendor and Part Number	Usage Notes
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.
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)
Optional: AMPure XP Kit (5 mL)	Beckman Coulter Genomics p/n A63880	Optional alternative to SureSelect Max Purification Beads (See Table 1)
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276	—
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	—
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 ring formation.
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	—
Agilent TapeStation system 4200/4150 TapeStation Instrument Consumables: 96-well sample plates 96-well plate foil seals 8-well tube strips 8-well tube strip caps D5000 ScreenTape D5000 Reagents	Agilent p/n G2991AA/G2992AA p/n 5042-8502 p/n 5067-5154 p/n 401428 p/n 401425 p/n 5067-5588 p/n 5067-5589	Automated electrophoresis system for library fragment sizing QC prior to downstream NGS.
NGS Library Quantification Kit (qPCR-based)	Various suppliers	Required for quantification of PCR-free whole genome libraries prior to NGS. Select a kit designed for Illumina library quantification.

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 an isolated work area, such as a laminar flow hood, and dedicated materials for PCR-free workflows when PCR amplification and product processing are performed in the same laboratory, to prevent contamination of samples or reagents with amplified DNA.
 - 2 Maintain clean work areas. Clean the surfaces that pose the highest risk of contamination daily using a 10% bleach solution, or equivalent.
 - 3 Use dedicated pipettors with nuclease-free aerosol-resistant tips.
 - 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

PCR-Free DNA Library Preparation using Sheared DNA Protocol

- Step 1. Prepare and qualify genomic DNA samples [12](#)
- Step 2. Fragment the DNA by Covaris shearing [14](#)
- Step 3. Prepare the ligation master mix [16](#)
- Step 4. Repair and dA-tail the DNA 3' ends [16](#)
- Step 5. Ligate full-length UDI adaptors [17](#)
- Step 6. Purify libraries using magnetic purification beads [18](#)
- Step 7. QC and quantify the libraries [21](#)

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 100 to 500 ng of input DNA, with adjustments to the recommended range of input DNA based on sample quality and intended NGS read length (see [Table 4](#)). 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, then ligated to full-length adaptors that include unique dual indexes (UDIs). The adaptor-ligated library fragments are purified using procedures for optimal size selection tailored to specific library types. The final prepared DNA libraries are qualified and quantified using methods appropriate for PCR-free whole genome sequencing (WGS).

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.

Table 4 DNA input amount recommendations

Sample type	NGS read length	Amount input DNA
High-quality (non-FFPE) samples	2 × 150 reads	100–500 ng
	2 × 250 reads	200–500 ng
FFPE samples	2 × 150 reads [*]	100–500 ng (at least 200 ng for low integrity samples; see page 13)

^{*} All FFPE sample libraries are prepared using 2 x 150 read-length NGS conditions.

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).

Before you begin, determine the appropriate SureSelect Max UDI Adaptor assignment for each sample. Use a different UDI number for each sample to be sequenced in the same lane. See [page 32](#) for sequences of the UDIs added to the library fragments in this step.

In preparation for the DNA shearing step on [page 15](#), 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.

Table 5 Reagents prepared before use in protocol

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect Max Library Preparation Module, PCR-free, 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 16
	T4 DNA Ligase (blue cap)	Place on ice just before use, invert to mix.	page 16
	End Repair-A Tailing Buffer (yellow cap or bottle)	Thaw on ice (may require >20 minutes) then keep on ice, vortex to mix.	page 17
	End Repair-A Tailing Enzyme Mix (orange cap)	Place on ice just before use, invert to mix.	page 17
SureSelect Max UDI Adaptors for WGS, PCR-free, ILM stored at –20°C	Adaptor with UDIs 1-8 in white 8-well strip OR Adaptor with UDIs 9-16 in black 8-well strip OR Adaptor with UDIs 1-96 in clear 96-well plate	Thaw the appropriate range of adaptors on ice then keep on ice, vortex to mix.	page 17
+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.	page 17

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.

The protocol requires 100–500 ng of input DNA for 2 x 150 read length NGS or 200–500 ng of input DNA for 2 x 250 read length NGS.

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 14.

Preparation and qualification of gDNA from FFPE samples

All FFPE samples use 2 x 150 read-length library preparation conditions.

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

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

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

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

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

- a Analyze a 1- μ L aliquot of each FFPE gDNA sample using the Genomic DNA ScreenTape assay. Follow the instructions provided in the [assay Quick Guide](#).
- b See [Table 6](#) for DIN score-based input DNA input guidelines.

Table 6 DNA input guidelines based on DNA Integrity Number (DIN)

DIN Score	DNA Input Guidelines*
DIN ≥ 3	100 ng to 500 ng DNA, based on Qubit Assay quantification
DIN < 3	200 ng to 500 ng DNA, based on Qubit Assay quantification

* Use the DNA concentration determined by the Qubit Assay, instead of the concentration determined by ScreenTape assay, to calculate the input DNA volume.

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 $\Delta\Delta Cq$ DNA integrity score and the precise quantity of amplifiable DNA in the sample, allowing direct normalization of DNA input for each sample.

- a Analyze a 1- μ L aliquot of each FFPE gDNA sample using the Agilent NGS FFPE QC Kit. Follow the instructions provided in the [kit user manual](#).
- b Use the $\Delta\Delta Cq$ score-based guidelines below (summarized in [Table 7](#)) to determine the appropriate input DNA amount and quantification method for your sample:

For all samples with $\Delta\Delta Cq$ DNA integrity score ≤ 1 (more intact FFPE DNA samples), use the Qubit-based gDNA concentration to determine the volume of input DNA needed for the 100–500 ng DNA.

For all samples with $\Delta\Delta Cq$ 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 the volume of input DNA needed for 200–500 ng DNA.

Table 7 DNA input guidelines based on $\Delta\Delta Cq$ DNA integrity score

$\Delta\Delta Cq$ Score	DNA Input Guidelines
$\Delta\Delta Cq \leq 1$	100 ng to 500 ng DNA, based on Qubit Assay quantification
$\Delta\Delta Cq > 1$	200 ng to 500 ng DNA, based on qPCR quantification

Proceed to [“Step 2. Fragment the DNA by Covaris shearing”](#) on page 14.

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- μ L shearing volume to produce DNA fragment sizes suitable for the intended NGS read length. See [Table 8](#) for a summary of protocol parameters for different sample and run types. Complete shearing instructions are provided on the following pages

Table 8 Covaris shearing protocol summary

Sample type	NGS read length	Amount input DNA	Shearing duration
High-quality (non-FFPE) samples	2 × 150 reads	100–500 ng	2 × 40 seconds
	2 × 250 reads	200–500 ng	2 × 10 seconds
FFPE samples	2 × 150 reads*	100–500 ng (at least 200 ng for low integrity samples)	480 seconds

* All FFPE sample libraries are prepared using the same conditions, including shearing for 480 seconds to generate fragment ends suitable for library construction. Initial DNA fragment size may impact the post-shear fragment size distribution and libraries 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 μ L Covaris microTUBE. Consult the manufacturer's recommendations for use of other Covaris instruments or sample holders to achieve similar DNA fragment sizes.

- 1 Prepare the DNA samples for the run by diluting 100–500 ng of gDNA in 1X Low TE Buffer to a final volume of 50 μ L. See [Table 8](#) for workflow-specific input amounts. 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.

CAUTION

The Covaris instrument shearing settings ([Table 9](#)) and shear duration used in the PCR-free WGS workflow differ from other SureSelect system workflows. Adhere to the instructions provided here; do not use protocols provided for other SureSelect kits.

- 2 Complete the DNA shearing steps below for each sample:
 - a Transfer the 50- μ L 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 × 150 read NGS	High-quality DNA for 2 × 250 read NGS	FFPE DNA for 2 × 150 read NGS
Duty Factor	2%	2%	2%
Peak Incident Power (PIP)	140	140	140
Cycles per Burst	200	200	200
Treatment Time	2 × 40 seconds (see two-round instructions below)	2 × 10 seconds (see two-round instructions below)	480 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 40 seconds or 10 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 40 seconds or 10 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 μ L) 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 with a pipette set to at least 80% of the mixture volume, 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 17](#).

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. Reagents may be depleted after fewer than 16 reactions when using smaller run sizes.

† 96 reaction Library Preparation Kits contain enough reagents for 4 runs of 24 samples each using the indicated excess volume. Reagents may be depleted after fewer than 96 reactions when using smaller run sizes.

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

- 4 Add 20 μ L of the end repair/dA-tailing master mix to each sample well containing 50 μ L of DNA fragments. Mix by pipetting up and down 15–20 times using a pipette set to 50 μ L or cap the wells and vortex at high speed for 5–10 seconds.
- 5 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and run the program in [Table 11](#).

NOTE

Remove the magnetic purification beads from cold storage and equilibrate to room temperature (RT) for use on [page 18](#). Keep beads at RT for least 30 minutes before use.

Step 5. Ligate full-length UDI adaptors

- 1 Once the thermal cycling program in [Table 11](#) reaches the final 4°C Hold step, transfer the samples to ice. Preprogram the cycler as shown in [Table 13](#) with heated lid off.

Table 13 Thermal cycler program for ligation (100 μ L vol)

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

- 2 To each end-repaired/dA-tailed DNA sample (approximately 70 μ L), add 25 μ L of the ligation master mix that was prepared on [page 16](#) and kept at room temperature. Mix by pipetting up and down at least 10 times using a pipette set to 70 μ L or cap the wells and vortex at high speed for 5–10 seconds. Briefly spin the samples.
- 3 Keeping the samples on ice, add 5 μ L of the assigned full-length SureSelect Max UDI Adaptor.

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 to the samples in separate addition steps, mixing after each addition, as directed above. Adding these components together may decrease kit performance.

- 4 Briefly spin the samples, then immediately place the plate or strip tube in the thermal cycler and run the program in [Table 13](#).

Step 6. Purify libraries using magnetic purification beads

NOTE

Before you begin, verify that the SureSelect Purification Beads or AMPure XP Beads were kept at RT for least 30 minutes before use in the protocol below.

The Whole Genome Library Preparation protocol uses different purification conditions optimized for specific library types as summarized in [Table 14](#). After reviewing the table, follow the detailed protocol steps provided below the table.

Table 14 Magnetic purification bead cleanup parameter summary

Step/Parameter	High-quality (fresh/fresh frozen) sample input DNA		FFPE sample input DNA
	2×150 read fragments	2×250 read fragments	2×150 read fragments
Rounds of purification required	Two (serial)	Two (serial)	One
Volume of fresh 70% ethanol to prepare (includes excess)	8-sample run: 7 mL 24-sample run: 21 mL	8-sample run: 7 mL 24-sample run: 21 mL	8-sample run: 3.5 mL 24-sample run: 10.5 mL
Round 1 bead volume	70 µL	60 µL	80 µL
Round 1 elution conditions	100 µL nuclease-free water	100 µL nuclease-free water	25 µL 1X Low TE Buffer
Round 2 bead volume	70 µL	60 µL	Not applicable
Round 2 elution conditions	25 µL 1X Low TE Buffer	25 1X Low TE Buffer	
Final eluted purified library transferred to fresh well	Approximately 24 µL in 1X Low TE Buffer	Approximately 24 µL in 1X Low TE Buffer	Approximately 24 µL in 1X Low TE Buffer

- 1 Prepare fresh 70% ethanol for use in washes in [step 8](#) and [step 24](#) (where applicable). See [Table 14](#) for volume required for your sample type and run size.
- 2 Using SureSelect Max Purification Beads or AMPure XP Beads equilibrated to RT, mix the beads well until homogeneous and consistent in color.
- 3 Move the sample strips from the thermal cycler to room temperature, then add the appropriate volume of bead suspension to each sample well containing approximately 100 µL of library:

For high-quality DNA libraries fragmented for 2 x 150 NGS: Add 70 µL of beads per well

For high-quality DNA libraries fragmented for 2 x 250 NGS: Add 60 µL of beads per well

For FFPE DNA libraries: Add 80 µL of beads per 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 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 the appropriate solvent to each sample well:
 - For high-quality DNA libraries:** add 100 µL of nuclease-free water per well
 - For FFPE DNA libraries:** add 25 µL of 1X Low TE Buffer per 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 each cleared supernatant to a fresh well and proceed as appropriate for your library type. You can discard the beads after collecting the supernatants.
 - For high-quality DNA libraries:** Transfer approximately 100 µL of each supernatant to a fresh strip well and proceed to [step 18](#) for the second round of purification.
 - For FFPE DNA libraries:** Transfer approximately 24 µL of each supernatant to a fresh strip well and keep the strip on ice. Proceed to [“Step 7. QC and quantify the libraries”](#) on page 21.

Round 2 purification for high-quality DNA input libraries only:

- 18 Mix the room-temperature purification beads well until homogeneous and consistent in color.
- 19 Add the appropriate volume of bead suspension to each sample well containing approximately 100 µL of library supernatant:
 - For high-quality DNA libraries fragmented for 2 x 150 NGS:** Add 70 µL of beads per well
 - For high-quality DNA libraries fragmented for 2 x 250 NGS:** Add 60 µL of beads per well

- 20 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.
- 21 Incubate the bead suspensions for 10 minutes at room temperature.
- 22 Put the plate or strip tube into a magnetic separation device. Wait for the solution to clear (approximately 5 minutes).
- 23 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.
- 24 Continue to keep the plate or strip tube in the magnetic stand while you dispense 200 μ L of fresh 70% ethanol in each sample well.
- 25 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 26 Repeat [step 24](#) and [step 25](#) once for a total of two washes.
- 27 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.

- 28 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.
- 29 Elute the library DNA by adding 25 μ L of 1X Low TE Buffer to each sample well.
- 30 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.
- 31 Incubate for 2–5 minutes at room temperature. Use of longer incubation times may improve recovery, especially for longer DNA fragments.
- 32 Put the plate or strip tube in the magnetic stand and leave until the solution is clear (up to 5 minutes).
- 33 Transfer approximately 24 μ L of each supernatant to a fresh strip well and keep the strip on ice. You can discard the beads after collecting the supernatants. Proceed to [“Step 7. QC and quantify the libraries”](#) on page 21.

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 7. QC and quantify the libraries

Library qualification with average fragment size determination

Analyze a sample of each library using the Agilent TapeStation system with the D5000 ScreenTape Assay (see the user guide link in [Table 15](#) for instructions).

Table 15 Library size qualification assay overview

Analysis platform	Assay used at this step	Amount of library sample to analyze
Agilent 4200/4150 TapeStation system	Agilent D5000 ScreenTape Assay	1 µL

Representative TapeStation electropherograms are provided in [Figure 2](#) and [Figure 3](#) to illustrate typical results.

Using the 100 bp to 3500 bp region of the electropherogram, determine the average fragment size. See [Table 16](#) for fragment size range guidelines.

Table 16 Expected library fragment size guidelines

Input type	NGS read length used to select fragmentation duration	Expected reported average fragment size (100–3500 bp region)
Intact DNA	2 × 150 reads	800 to 1300 bp
	2 × 250 reads	1100 to 1600 bp
FFPE DNA	2 × 150 reads	400 to 1200 bp

Adjustments to reported average fragment sizes

The full-length adaptors used for PCR-free library prep contain single-stranded regions that can retard migration during TapeStation electrophoresis, resulting in a larger apparent size profile compared to the actual library fragment size distribution. The reported average fragment size values should be adjusted before use in qPCR-based library quantification using the following conversion:

$$\text{Adjusted average fragment size (bp)} = [0.98 \times \text{reported average size (bp)}] - 137 \text{ bp}$$

Proceed to [page 23](#) for qPCR quantification guidelines.

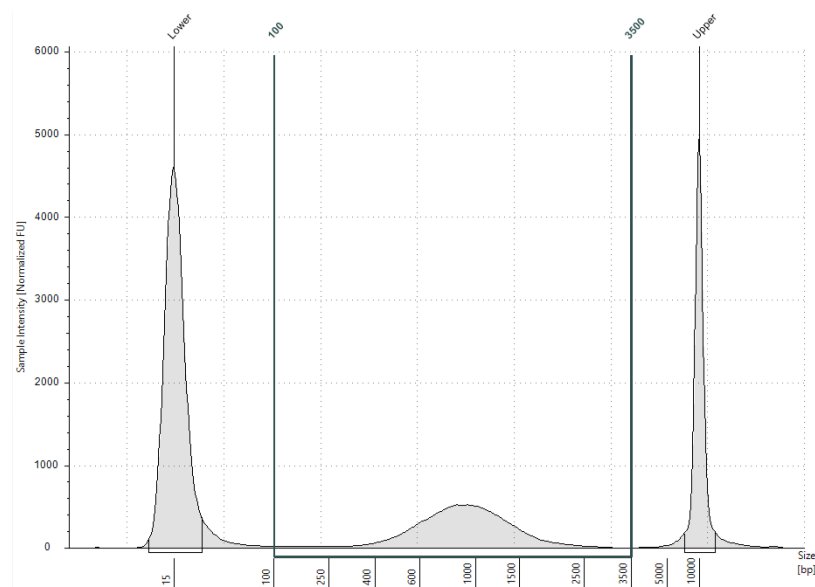


Figure 2 Library prepared from 100 ng of a high-quality gDNA sample, fragmented using 2x150 read length conditions.

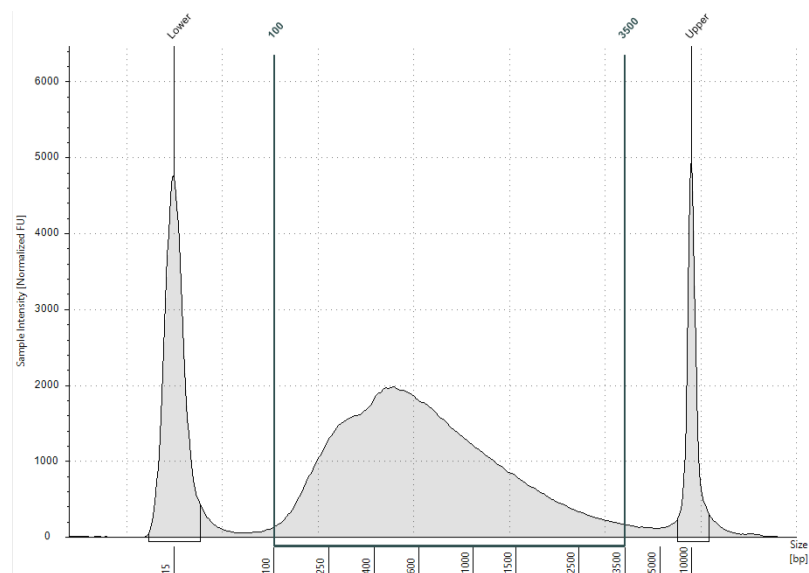


Figure 3 Library prepared from 200 ng of a medium-quality FFPE gDNA sample, fragmented using 2x150 read length conditions.

qPCR-based library quantification

Determine the concentration of each library using an appropriate qPCR-based NGS Library Quantification Kit designed to quantify fragments containing Illumina adaptor sequences.

Follow the manufacturer's protocols for standard curve generation and pM library concentration determination based on cycle threshold (Ct) values. The approximate library concentration from the TapeStation assay can be used to determine the appropriate library dilution scheme for qPCR quantification.

The qPCR quantification system should include instructions for correcting the pM library concentration value from the qPCR assay based on the relative lengths of the qPCR Standard and the library fragments. In these correction calculations, use the **adjusted** average fragment size (bp) determined for each library as calculated on [page 21](#).

If you have not already corrected the qPCR-determined library concentration values based on fragment size, use the formula below for this correction:

Adjusted [Library] = Measured [Library] × (qPCR Standard bp/Adjusted average library fragment size bp)

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

3

Appendix: NGS Guidelines

Library Pooling Guidelines for Multiplex WGS [25](#)

Sequencing Setup and Run Guidelines [26](#)

Analysis Pipeline Guidelines [27](#)

This appendix provides guidelines for SureSelect Max PCR-free Whole Genome NGS using the Illumina platform.

Library Pooling Guidelines for Multiplex WGS

The SureSelect Max whole genome libraries are ready for pooling for multiplex WGS. Libraries multiplexed for sequencing in the same lane should be combined into a normalized pool using a plexity, concentration, and volume appropriate for your sequencing platform and research design.

Pool plexity: The number of indexed libraries that may be multiplexed is determined by the output specifications of the sequencer, 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.

Normalized pool concentration: The required normalized pool concentration is determined by the optimal final loading concentration for PCR-free whole genome libraries for your selected sequencing platform and any pre-loading dilution steps in your NGS workflow.

For example, for NovaSeq 6000 Xp workflow runs, if the optimized final loading concentration is 100 pM, normalized library pools should be prepared at 0.5 nM to allow for pre-loading dilutions. Consult Illumina’s resources for more information for your NGS system.

NOTE Accurate optimized loading concentrations can vary for libraries quantified with different qPCR quantification kits. The optimal final loading concentration should be titrated for the specific combination of sequencing and quantification systems used in your workflow.

An example of pooling conditions optimized for Agilent’s SureSelect Max ILM PCR-free WGS libraries for one combination of sequencing and quantification systems is provided in [Table 17](#). Optimal conditions for your quantification and NGS workflow may vary.

Table 17 Example Pool Concentration and Volume Determinations

qPCR Quantification Kit Manufacturer	Illumina Sequencing System	Desired Final Loading Concentration	Required Library Pool Concentration*
Takara	NovaSeq 6000 (Xp Workflow)	50–150 pM	0.25–0.75 nM

* For the NGS system in this example, libraries need to be pooled at a concentration 5× the intended final sequencer loading concentration (seeding concentration).

Pool volume: Consult Illumina’s support resources to determine the required pool volume for your sequencing platform and flow cell.

Normalized library pooling instructions

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. Then combine equal volumes of all diluted samples to create the final NGS pool at the final volume required for your platform.

For example, if multiplexing 4 indexes with 0.5 nM pool concentration and 30 µL pool volume, dilute all libraries to 0.5 nM then combine 7.5 µL of each library.

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 final volume required for your platform. 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 DNA in the pool

$\#$ is the number of indexes, and

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

Table 18 shows an example of the amount of 4 indexed samples of different concentrations and amount of Low TE needed for a final volume of 30 μL at 0.5 nM DNA.

Table 18 Example pooling volume calculations

Component	V(f)	C(f)	# of indexes	C(i)	Volume to use (μL)
Sample 1	30 μL	0.5 nM	4	0.5 nM	7.5
Sample 2	30 μL	0.5 nM	4	0.75 nM	5.0
Sample 3	30 μL	0.5 nM	4	1.0 nM	3.75
Sample 4	30 μL	0.5 nM	4	1.5 nM	2.5
Low TE					11.25

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 [Figure 4](#)) including a unique pair of 8-bp P5 and P7 indexes suitable for Illumina sequencing platforms. See [“SureSelect Max Full-length Adaptor UDI Information”](#) on page 30 for additional index information.

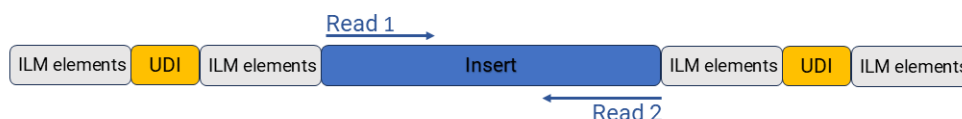


Figure 4 Content of SureSelect Max ILM Whole Genome PCR-free sequencing libraries. Each fragment contains one target insert (blue), along with Illumina paired-end sequencing elements (gray) and unique dual indexes (gold).

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. Final loading concentration (or seeding concentration) should be optimized for your selected sequencing platform and qPCR quantification kit (see [page 25](#)). Follow Illumina's recommendation for a PhiX 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 19](#) showing example settings for 2 x 150 bp sequencing.

Table 19 Run settings for 2 x 150 bp sequencing

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

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

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

- Each of the sample-level indexes (i7 and i5) requires an 8-bp index read. For complete index sequence information, see [page 32](#).
- 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 additional SureSelect Max for ILM library sequencing guidance, 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 PCR-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 be trimmed using Illumina's demultiplexing software by specifying adaptor trimming in the sample sheet or using a suitable tool designed to trim the demultiplexed reads. Use the following sequences for adaptor trimming.

Read 1: AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

Read 2: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
- Align the trimmed reads using a suitable tool such as BWA-MEM.

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

4 Reference

Reagent Kit Contents [29](#)

SureSelect Max Full-length Adaptor UDI Information [30](#)

Troubleshooting Guide [34](#)

Quick Reference Protocol: PCR-free Whole Genome Library Prep [36](#)

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 PCR-free whole genome DNA library preparation for Covaris-sheared DNA uses the kits listed in [Table 20](#). Detailed contents of the multi-part component kits are shown in [Table 21](#) and [Table 22](#).

Table 20 Kits for SureSelect Max PCR-free WGS Library Preparation with Covaris shearing

Reagent Kit Modules	Part Number		Storage Condition
	16 Reactions	96 Reactions	
SureSelect Max Library Preparation Module, PCR-free	5280-0150	5280-0151	–20°C
SureSelect Max UDI Adaptors for WGS, PCR-free, ILM	5280-0098 (Index 1-16)	5280-0099 (Index 1-96)	–20°C
SureSelect Max Purification Beads	5282-0225	5282-0226	+4°C

Component kit details

Table 21 SureSelect Max Library Preparation Module, PCR-free content

Kit Component	16 Reaction Kit (p/n 5280-0150)	96 Reaction Kit (p/n 5280-0151)
End Repair-A Tailing Enzyme Mix	tube with orange cap	tube with orange cap
End Repair-A Tailing Buffer	tube with yellow cap	bottle
T4 DNA Ligase	tube with blue cap	tube with blue cap
Ligation Buffer	tube with purple cap	bottle

Table 22 SureSelect Max UDI Adaptors for WGS, PCR-free for ILM content

Kit Component	16 Reaction Kit Format	96 Reaction Kit Format
SureSelect Max UDI Adaptors for WGS, PCR-free, ILM*	White 8-well strip tube (index pairs 1-8)	Clear 96-well plate (index pairs 1–96)
	AND	
	Black 8-well strip tube (index pairs 9-16)	

* See [page 30](#) for index strip and plate position maps; see [page 32](#) and [page 33](#) for index pair sequence information.

CAUTION

The SureSelect Max UDI Adaptors 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.

SureSelect Max Full-length Adaptor UDI Information

The SureSelect Max ILM UDI Adaptors for PCR-free WGS are provided in pre-combined index pairs. Each adaptor UDI well includes a unique 8-bp P5 index paired with a unique 8-bp P7 index, resulting in uniquely dual-indexed NGS libraries after adaptor ligation. Each well of the provided 8-well strip tubes contains a single-use aliquot of a specific index pair. Adaptor UDI strip and plate maps are provided below.

Nucleotide sequences of the indexes are provided in [Table 24](#) on page 32 and [Table 25](#) on page 33. Index sequences can also be obtained by downloading the [SureSelect Max UDI Adaptors for ILM WGS 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 24](#) and [Table 25](#) 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.

NOTE

The indexes in the SureSelect Max ILM UDI Adaptors are shared with indexes used in other Agilent NGS library preparation systems. Different index numbers can have the same index sequence when comparing across these systems.

Before combining the SureSelect Max PCR-free WGS libraries with other types of SureSelect or Avida libraries in the same sequencing run, compare the **index sequences** to make sure that each index pair sequence is unique. Do not pool and run libraries together if they share index sequences.

Adaptor index pair strip tube and plate maps

SureSelect Max UDI Adaptors for WGS, PCR-free, ILM (UDI 1–16) are supplied in a set of two 8-well strip tubes as detailed below.

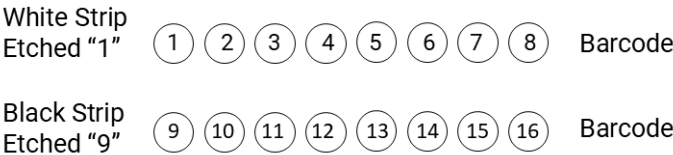


Figure 5 Map of the provided UDI Adaptor strips

The white strip contains adaptors with index pairs 1–8, with pair #1 supplied in the well proximal to the numeral **1** etched on the strip's plastic end tab.

The black strip contains adaptors with index pairs 9–16, with pair #9 supplied in the well proximal to the numeral **9** etched on the strip's plastic end tab.

Pierce the foil seal of the appropriate well with a pipette tip just before pipetting the solution.

Table 23 Plate map for SureSelect Max UDI Adaptors for WGS, PCR-free, ILM 1-96, provided in clear 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

SureSelect Max full-length adaptor index sequences

Index sequences can also be obtained by downloading the [SureSelect Max UDI Adaptors for ILM WGS Index Sequence Resource](#) Excel spreadsheet from Agilent.com.

Table 24 SureSelect Max full-length adaptor UDIs 1–48, provided in clear 96-well plate (1–16 also provided in strip tubes)

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
1	A01	CGTTCGTC	GCAGTTAG	CTAACTGC	25	A04	CGCGCAAT	CAGAGCAG	CTGCTCTG
2	B01	AATGCTAG	ATACTCGC	GCGAGTAT	26	B04	GCCTTCAT	AACGGTCA	TGACCGTT
3	C01	AAGTGGAC	TACGTAGC	GCTACGTA	27	C04	GATGCCAA	TTAGTTGC	GCAACTAA
4	D01	GCACACAT	AATACGCG	CGCGTATT	28	D04	CGTCTGTG	TTAACGTG	CACGTTAA
5	E01	AGAACCGT	TACGGTTG	CAACCGTA	29	E04	GTGCGCTT	CATCGACG	CGTCGATG
6	F01	TGCGCTCT	GTCGCCTA	TAGGCGAC	30	F04	GTACGGAC	GCACGATG	CATCGTGC
7	G01	CTGAGCTA	GCACAGTA	TACTGTGC	31	G04	AACGCATT	ATAGTGAC	GTCACTAT
8	H01	TAGGACAT	CATCGGAA	TTCCGATG	32	H04	ATATTCCG	GACAATTG	CAATTGTC
9	A02	TCAGCATC	AAGGAGCG	CGCTCCTT	33	A05	GATCGCCT	TGTATCAC	GTGATACA
10	B02	CTTGCGAT	CTGAGCTA	TAGCTCAG	34	B05	TCTGAGTC	ATCATGCG	CGCATGAT
11	C02	CGTAGTTA	GTAGACTG	CAGTCTAC	35	C05	TCTACCTC	ATATTCCG	CGGAATAT
12	D02	GTAGTCAT	AACTCTTG	CAAGAGTT	36	D05	CGCTTCCA	TCGAACTG	CAGTTCGA
13	E02	TCCGAACT	TCATACCG	CGGTATGA	37	E05	GAGTGCCT	TCGCGTCA	TGACGCCA
14	F02	CGTCAGAT	CGTAGTTA	TAACTACG	38	F05	GAGGCTCT	CTAGTGTA	TACACTAG
15	G02	ATGGTAGC	GTCGATCG	CGATCGAC	39	G05	AACACCGA	CGATGTGC	CGACATCG
16	H02	TCGCCTGT	TCTGAGTC	GA CTCAGA	40	H05	CGAATTCA	TTGGCATG	CATGCCAA
17	A03	AGCTACAT	GATCCATG	CATGGATC	41	A06	AGATACGA	CATACTTG	CAAGTATG
18	B03	GAAGGTAT	CGACTACG	CGTAGTCG	42	B06	TGTCGTCG	GTTATCGA	TCGATAAC
19	C03	AATGGAAC	GTTGCGGA	TCCGCAAC	43	C06	GCCAACAA	GTAATACG	CGTATTAC
20	D03	AGCAGTGA	CACAGGTG	CACCTGTG	44	D06	CATCAGTC	TTATCTCG	CGAGATAA
21	E03	TCAGCTCA	TATGCCGC	GCGGCATA	45	E06	CTAGTGTA	AACAGCCG	CGGCTGTT
22	F03	CAACCGTG	AAGTGGAC	GTCCACTT	46	F06	CAGTCATT	TCGAAGGA	TCCTTCGA
23	G03	CAGCAATA	ATTAGGAG	CTCCTAAT	47	G06	GA CTTGAC	GCATCATA	TATGATGC
24	H03	TGGATTGA	TAAGAGTG	CACTCTTA	48	H06	GTCCACCT	AGTTGCCG	CGGCAACT

Table 25 SureSelect Max full-length adaptor UDIs 49–96, provided in clear 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
49	A07	TCTGTCAG	CGTAAGCA	TGCTTACG	73	A10	GCGAATTA	CTTGATCA	TGATCAAG
50	B07	CTTCACGT	GAATCGCA	TGCGATTG	74	B10	GTCGAAGT	CAGTGAGC	GCTCACTG
51	C07	CTTGATCA	CAATGCTG	CAGCATTG	75	C10	CATTGCAT	CGACGGTA	TACCGTCG
52	D07	TGCACATC	GAGTGTAG	CTACACTC	76	D10	TTCCAACA	GCTTAGCG	CGCTAAGC
53	E07	GCATGCCT	CGTAACTG	CAGTTACG	77	E10	AATACGCG	CAGGTCTG	CAGACCTG
54	F07	CACAACTT	AGGTGAGA	TCTCACCT	78	F10	TCTAGAGC	GATAGAGA	TCTCTATC
55	G07	CTGCCTTA	GCATGGAG	CTCCATGC	79	G10	TAAGAGTG	AGAAGACG	CGTCTTCT
56	H07	TCGATACA	TTAGTGCG	CGCACTAA	80	H10	AGTCTTCA	CAGTATCA	TGATACTG
57	A08	CAATAGAC	ATGACTCG	CGAGTCAT	81	A11	CAAGTTAC	TATTCCTG	CAGGAATA
58	B08	CGCCAATT	ATGGTAGC	GCTACCAT	82	B11	CGTAACTG	TGTCGTCTG	CGACGACA
59	C08	TGCCTCGA	CAGTTGCG	CGCAACTG	83	C11	GCTTAGCG	ATAAGCTG	CAGCTTAT
60	D08	GAGCACTG	GAATTGTG	CACAATTC	84	D11	TCAACTGC	GCTACGCA	TGCGTAGC
61	E08	GCCGATAT	AGACCTGA	TCAGGTCT	85	E11	AAGAGACA	TGCTTACG	CGTAAGCA
62	F08	CACAGACT	TCTGTCAG	CTGACAGA	86	F11	CGGTCTCT	GAGATCGC	GCGATCTC
63	G08	CTTAAGAC	GA CTGACG	CGTCAGTC	87	G11	TCGAGCAT	CGTTAACG	CGTTAACG
64	H08	AAGTACTC	GATAGGAC	GTCCTATC	88	H11	TCACAAGA	TTGTGTAC	GTACACAA
65	A09	TCGACAAC	CTCTCGTG	CACGAGAG	89	A12	TTGGATCT	TGCCGTTA	TAACGGCA
66	B09	GCCAGTAG	GTTGAGC	GCTCGAAC	90	B12	AGAGTAAG	GAGACACG	CGTGTCTC
67	C09	ATACCTAC	TCCTTGAG	CTCAAGGA	91	C12	AACCTAAC	CGTCTGTG	CACAGACG
68	D09	CATGAATG	TCTCCTTG	CAAGGAGA	92	D12	AGTACGTT	AGAGTAAG	CTTACTCT
69	E09	CACACATA	CTCGTACG	CGTACGAG	93	E12	GTTTCATTC	GTCGGTAA	TTACCGAC
70	F09	TCCTGAGA	G TAGAGCA	TGCTCTAC	94	F12	AACTCTTG	AAGCCATC	GATGGCTT
71	G09	ATTGAGCT	TCACTGTG	CACAGTGA	95	G12	TCCAGACG	TGGCTTAG	CTAAGCCA
72	H09	TCTATGCG	GCGGTATG	CATACCGC	96	H12	CAATCCGA	CACATTTCG	CGAATGTG

Troubleshooting Guide

If yield of libraries is low

- ✓ Use 1X Low TE Buffer (10 mM Tris-HCl pH 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.
- ✓ 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 ([page 13](#)) to determine the precise quantity of library-compatible 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 19](#) and [step 28](#) on [page 20](#)). Monitor the bead pellets during the drying incubation frequently 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

- ✓ The PCR-free WGS workflow uses different shearing conditions than other SureSelect system workflows. Adhere to the instructions provided. Verify that the parameters provided in [Table 9](#) on [page 15](#) were used in the run. Check the Covaris instrument duty factor and PIP settings, and verify use of the shearing duration appropriate for your sample type and read length.
- ✓ Verify that gDNA samples were diluted for shearing in 1X Low TE Buffer and not in water. Shearing in water may reduce the average library fragment length.
- ✓ DNA fragment size selection during magnetic bead purification depends upon using the correct ratio of sample to 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 for your sample type.
- ✓ For libraries prepared from high-quality DNA (from fresh/fresh-frozen samples), the average library fragment size can be increased by adjusting the ratio of bead suspension to DNA sample volume in the library purification procedure on [page 30](#) as detailed below. **Note that this protocol modification will also decrease the overall library yield.**
 - For 2x150 NGS the standard protocol uses beads at 0.7X the library DNA sample volume. To increase the average fragment size in the library, decrease the bead volume ratio to 0.6X (with 60 µL of purification bead suspension added to each 100 µL sample). Use the adjusted ratio in both rounds of purification.
 - For 2x250 NGS the standard protocol uses beads at 0.6X the library DNA sample volume. To increase the average fragment size in the library, decrease the bead volume ratio to 0.5X (with 50 µL of purification bead suspension added to each 100 µL sample). Use the adjusted ratio in both rounds of purification.
- ✓ FFPE DNA 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 13](#).

If library concentration values reported by qPCR assay versus TapeStation assay are significantly different

- ✓ Accurate quantification of SureSelect Max PCR-free WGS library fragments prior to NGS requires use of a qPCR-based assay, as described on [page 23](#), in order to specifically quantify the NGS adaptor-containing DNA fragments. This concentration value may significantly differ from the value reported by automated electrophoresis, which includes all DNA fragments in the selected size range such as unmodified input DNA fragments. SureSelect workflows that include a PCR step can use automated electrophoresis for quantification prior to NGS due to the selective PCR amplification of the adaptor-containing fragments in library mixtures.

Quick Reference Protocol: PCR-free Whole Genome Library Prep

An abbreviated protocol summary is provided below for experienced users. Use the complete protocol on [page 11](#) to [page 23](#) until you are familiar with all of the protocol details.

Step	Summary of Conditions
Prepare and qualify DNA samples	Dilute 100–500 ng gDNA samples in 50 µL 1X Low TE Buffer. See Table 4 on page 10 for DNA input amount requirements for specific workflows.
Shear DNA samples	Shear 50 µL DNA samples using Covaris (Table 26) > 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 of assigned SureSelect Max UDI Adaptor> mix> spin. Incubate in thermal cycler: 30 min @ 20°C, Hold @ 4°C
Purify library DNA (Round 1) All libraries	For 2x150 high-quality libraries: 100 µL library DNA + 70 µL purification bead suspension For 2x250 high-quality libraries: 100 µL library DNA + 60 µL purification bead suspension For FFPE libraries: 100 µL library DNA + 80 µL purification bead suspension Mix> incubate 10 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute DNA in using appropriate solvent conditions for your library type: High-quality DNA libraries: 100 µL nuclease-free H ₂ O FFPE DNA libraries: 25 µL of 1X Low TE Buffer Add solvent> mix> incubate 2-5 minutes> collect beads> transfer library in supernatant to fresh well. For high quality DNA libraries proceed to round 2 purification steps below. FFPE library prep is complete; proceed to final QC steps.
Purify library DNA (Round 2) High-quality DNA libraries only	For 2x150 high-quality libraries: ~100 µL DNA supernatant + 70 µL purification bead suspension For 2x250 high-quality libraries: ~100 µL DNA supernatant + 60 µL purification bead suspension Mix> incubate 10 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute DNA in 25 µL of 1X Low TE Buffer > mix> incubate 2-5 minutes> collect beads> transfer library in supernatant to fresh well.
Quantify and qualify DNA	QC by TapeStation (see page 21 for size adjustment recommendations). Quantify library DNA for NGS using qPCR.

Table 26 Shearing conditions for PCR-free WGS libraries

Duty factor	Peak Incident Power (PIP)	Shearing duration according to sample type/intended read length		
		Input DNA	NGS Read Length	Shearing Time
2%	140	High quality DNA	2x150 reads	2 × 40 seconds
			2x250 reads	2 × 10 seconds
		FFPE-derived DNA	2x150 reads	480 seconds

In This Book

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

