Preparing SureSelect Max Libraries for Long-read Sequencing Workflows

Recommended SureSelect Max protocol adaptations for long-read NGS using sheared DNA

Technical Guide

Version A0, February 2026

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Notices

Manual Part Number

G9963-90001

Edition

Version A0, February 2026

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In this Guide...

This technical guide provides recommendations for adapting Agilent SureSelect Max DNA protocols to a long-read NGS library prep workflow using mechanically sheared DNA and fast hybridization for target enrichment. The provided recommendations are based on preliminary optimization studies; Agilent has not validated the performance of the recommended procedures and no adjustments were made by PacBio or ONT to their technologies or reagents.

1 Introduction to Long-read NGS using SureSelect Max Libraries

This section provides an overview of long-read NGS workflows and how Agilent SureSelect Max DNA reagents and protocols can be adapted for use in long-read NGS.

Overview of Long-read NGS

Targeted enrichment coupled with long-read sequencing has emerged as a powerful tool for high-resolution genomic analysis, enabling the detailed interrogation of specific loci that are challenging to assess with short-read technologies. By focusing sequencing efforts on regions of interest — such as disease-associated genes (e.g., BRCA1/2), complex immune loci (e.g., HLA), or structurally variable regions — this approach maximizes data quality while optimizing cost-efficiency. Long-read sequencing platforms, including PacBio HiFi and Oxford Nanopore Technologies (ONT), provide critical advantages by delivering reads spanning kilobases to megabases, facilitating the resolution of repetitive elements, phasing of haplotypes, and accurate detection of structural variants (SVs) and fusion genes. These capabilities are particularly valuable in clinical diagnostics, where comprehensive variant detection is essential for precision oncology and rare disease research, as well as in agricultural genomics for breeding applications and evolutionary studies of highly polymorphic gene families.

This publication highlights the compatibility and recommended workflow adjustments of SureSelect Max DNA library preparation and target enrichment with SureSelect panels for sequencing on the PacBio and ONT systems.

Using this Technical Guide

This Technical Guide describes recommendations for adapting published SureSelect Max DNA protocols to a long-read NGS workflow. An overview of the workflow is shown in Figure 1 on page 7, divided into the protocol segment sections of this publication. The recommendations are intended to guide use of Agilent SureSelect Max reagent kits to prepare long DNA libraries for further conversion to long-read NGS libraries using ONT or PacBio conversion reagent kits and protocols.

Detailed protocols for the SureSelect Max DNA library prep and Fast Hyb target enrichment workflow segments can be found on the Agilent website using the links below:

- SureSelect Max Library Prep using Sheared DNA
- SureSelect Max Fast Hybridization

This guide provides an abbreviated version of the SureSelect Max protocol steps, highlighting the protocol changes recommended for long DNA fragment libraries. New SureSelect Max users should read and understand the full protocols linked above before using the abbreviated protocols in this guide.

This guide also provides guidelines for performing the additional long-read NGS workflow steps not included in the published SureSelect Max protocols:

- Long-fragment shearing using Covaris g-TUBE devices
- Size-selection of sheared DNA during library preparation
- Final SureSelect Max library conversion to long-read NGS libraries using PacBio or ONT reagent kits and protocols
- Pre-capture and post-capture QC on the Agilent Femto Pulse or TapeStation platform

The SureSelect Max protocol steps were evaluated and optimized for long-read performance by Agilent as a starting point for customer-developed SureSelect Max DNA library long-read applications.

Overview of Workflow Segments

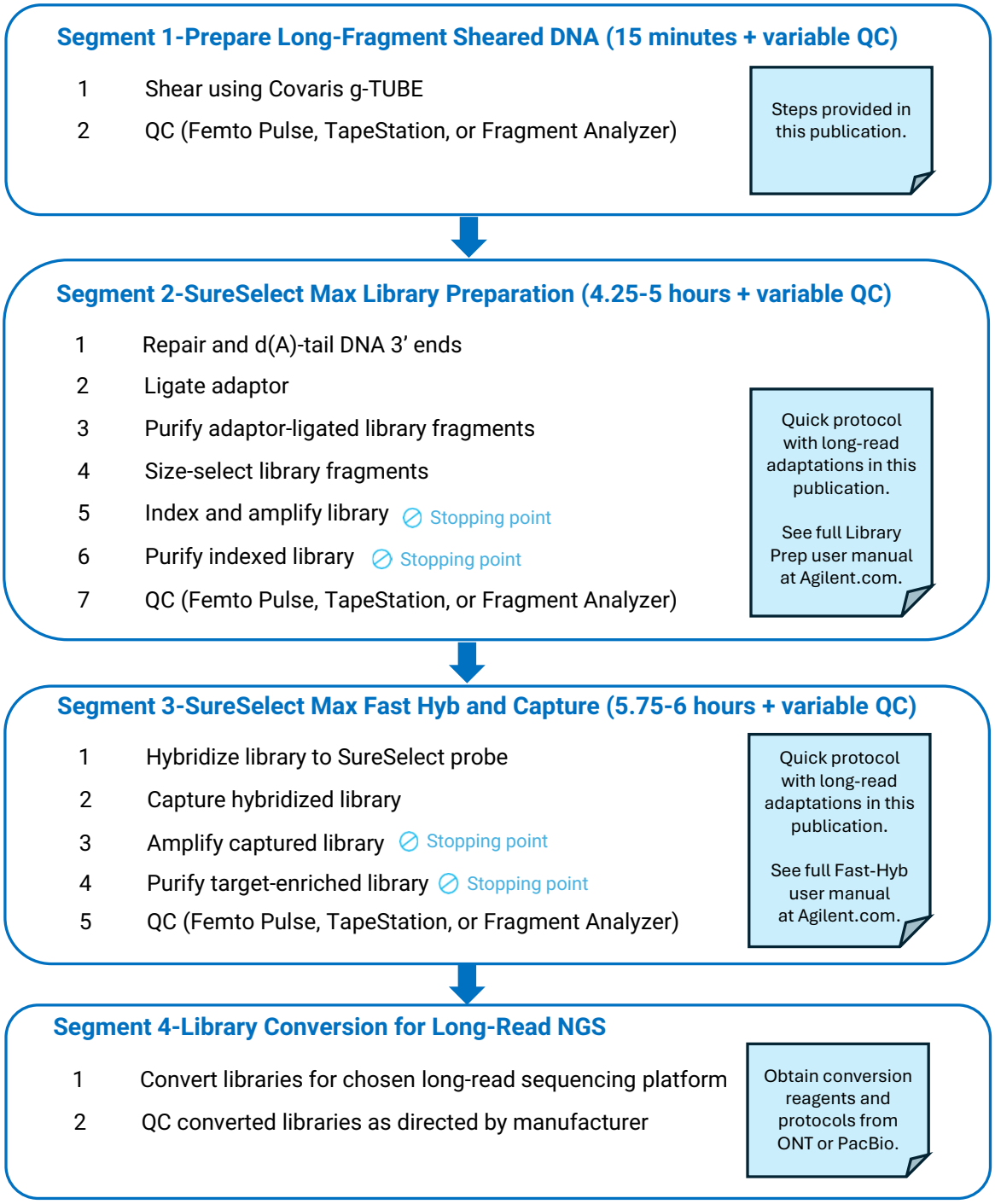

Figure 1 Overview of the long-read library construction workflow. The workflow is divided into four (4) segments covered in separate protocol sections in this publication.

Materials Required

SureSelect Max Modules Used in the Workflow

Table 1 SureSelect Max Kits for DNA Library Prep with Mechanical Shearing and Fast-Hyb Target Enrichment

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 XT HS Probe	See Agilent.com for a lists of pre-designed and custom probe options. Choose 90 min Hyb/HS design probes for use with the SureSelect Max Fast Hybridization modules.	
SureSelect Max Fast Hyb Kit*	G9689A	G9689B
SureSelect Max Blockers and Primers Kit for ILM	G9699A	G9699B
SureSelect Max Purification Beads†	G9962A (5 mL)	G9962B (30 mL)

* Includes SureSelect Streptavidin Beads. Separate purchase not required.

† May be substituted with AMPure XP beads (see Table 3 for ordering information). See page 24 for minor protocol adjustments.

QC Platform Options Used in the Workflow

Table 2 QC Platform Options for the Long-read Workflow

Description	Agilent Part Number
Agilent Femto Pulse System Genomic DNA 165 kb Kit (275 samples)	M5330AA FP-1002-0275
Agilent 4200/4150 TapeStation Systems Consumables: 96-well sample plates 96-well plate foil seals 8-well tube strips 8-well tube strip caps Genomic DNA ScreenTape Genomic DNA Reagents	G2991BA/G2992AA p/n 5042-8502 p/n 5067-5154 p/n 401428 p/n 401425 p/n 5067-5365 p/n 5067-5366
Agilent 5200/5300/5400 Fragment Analyzer Systems HS Large Fragment 50 kb Kit	M5310AA/M5311AA/M5312AA p/n DNF-464-0500

Additional Materials Used in the Workflow

See Table 3 for additional reagents and equipment used for SureSelect Max DNA long fragment library preparation.

Consult the product documentation provided by PacBio or ONT for a list of materials required for long-read library conversion and sequencing.

Table 3 Ordering Information for Additional Reagents and Equipment

Description	Vendor and Part Number	Usage Notes
Covaris g-TUBEs	Covaris p/n 520079 (10 pk)	Covaris single use shearing device. Requires use of supported microcentrifuge.
Microcentrifuge	Eppendorf microcentrifuge, model 5424 or equivalent	Select a microcentrifuge with conditions optimized for the Covaris g-TUBE shearing procedure (see the Covaris g-TUBE User Manual).
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)
Thermal Cyclers (2) with 96-well, 0.2 mL blocks	Various suppliers	Hybridization protocols require two thermal cyclers to complete a reagent pre-warming step during certain sample incubation steps.
Plasticware compatible with the selected thermal cycler: 8-well strip tubes Tube cap strips	Consult the thermal cycler manufacturer's recommendations	Runs using 1–3 strip tubes per run are recommended to facilitate efficient sample handling and to minimize the number of required thermal cyclers. Runs may also be completed using 96 well plates.
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
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 device configured to collect the particles in ring formation.
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent	—
1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent	Use for gDNA sample dilution prior to shearing and certain sample dilution and elution steps.
10 mM Tris, pH8	General laboratory supplier	Use for purification bead dilution prior to sheared fragment size selection.
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	—
Multichannel and single channel pipettes; sterile, nuclease-free aerosol barrier pipette tips; vortex mixer; ice bucket, powder-free gloves	General laboratory supplier	—
Optional: AMPure XP beads	Beckman Coulter Genomics	Optional alternative to SureSelect Max Purification Beads (See Table 1 on page 8)

2 SureSelect Max for Long-read Sequencing Workflow Protocol Guidelines

This section provides guidelines for preparation of target-enriched SureSelect Max DNA libraries for use in Oxford Nanopore Technologies (ONT) or Pacific Biosciences (PacBio) long-read sequencing workflows.

See Figure 1 on page 7 for an overview of the workflow.

Segment 1: Prepare Long-fragment Sheared DNA

This section provides the recommended procedures for preparing long sheared DNA fragments suitable for long-read NGS library prep.

Before you begin, prepare high-quality genomic DNA (gDNA) from fresh or fresh-frozen samples using a suitable purification system, such as Qiagen's QIAamp DNA Mini Kit, following the manufacturer's protocol. Agilent recommends performing QC on the gDNA sample prior to shearing to help ensure that the sample is high quality and has the expected size profile. The Femto Pulse system is the preferred QC platform for resolving intact, high-molecular weight gDNA over 20 kb.

The library preparation protocol uses 200 ng of sheared DNA in a 50 µL sample volume.

Step	Summary of Conditions
1.1 Prepare gDNA samples for g-TUBE shearing	**1** Prepare samples for shearing by diluting 600 ng of gDNA with 1X Low TE to 150 µL final volume. These shearing sample conditions use the shearing volume recommended by Covaris and result in 200 ng sheared DNA per 50 µL sample volume. If <600 ng gDNA is available, see *Troubleshooting* on page 29. **2** Bring the gDNA samples to room temperature (RT) before shearing by leaving samples on the benchtop 10 minutes.
1.2 Shear the DNA for long read-sized fragments	Shear the room RT genomic DNA samples in Covaris g-TUBEs using Covaris-recommended conditions for a target average fragment size of 10 kb. Conditions are summarized below: **1** Add 150 µL of DNA solution to Covaris g-TUBE. **2** Spin using the centrifugation speed and duration recommended by Covaris for your centrifuge. For the Eppendorf 5424 centrifuge, spin at 6000 RPM for 1 minute. The entire sample should be located at the bottom of the g-TUBE. If the g-TUBE retains any residual liquid, spin for an extra 30–60 seconds or until all of the sample has passed through the g-T. **3** Flip the g-TUBE over and repeat the centrifugation procedure in step 2. **4** Recover the entire sheared DNA solution (typically 150 µL) from the g-TUBE cap and transfer to a DNA LoBind tube. Store sheared DNA at +4°C.
1.3 QC sheared DNA	Analyze sheared DNA fragment size distribution as detailed in "Sheared DNA QC."

Sheared DNA QC

Analyze an aliquot of the sheared DNA using one of the platforms listed in Table 4. Follow the instructions in the linked user guide. The sheared DNA samples may require dilution before analysis to ensure that the DNA concentration is in the linear range of the assay.

Table 4 Post-capture library analysis options

Analysis platform	Assay used at this step	Link to assay instructions	Amount of sample to analyze
Agilent Femto Pulse system	Genomic DNA 165 kb Kit	Genomic DNA 165 kb Kit Quick Guide for the Femto Pulse	2 µL
Agilent 4200/4150 TapeStation systems	Genomic DNA ScreenTape	Genomic DNA ScreenTape Assay Quick Guide	1 µL
Agilent 5200/5300/5400 Fragment Analyzer systems	HS Large Fragment 50 kb Kit	HS Large Fragment 50 kb Kit Quick Guide	2 µL

Representative Femto Pulse, TapeStation, and Fragment Analyzer electropherograms are provided in Figure 2, Figure 3 and Figure 4 to illustrate typical results. Using the 0.5 kb to 60 kb region of the electropherogram, determine the average fragment size and the DNA concentration. See Table 5 for fragment size distribution guidelines.

Table 5 Expected sheared gDNA fragment size guidelines

Sample analyzed	Expected average fragment size (0.5–60 kb region)
g-TUBE sheared DNA	9 to 13 kb

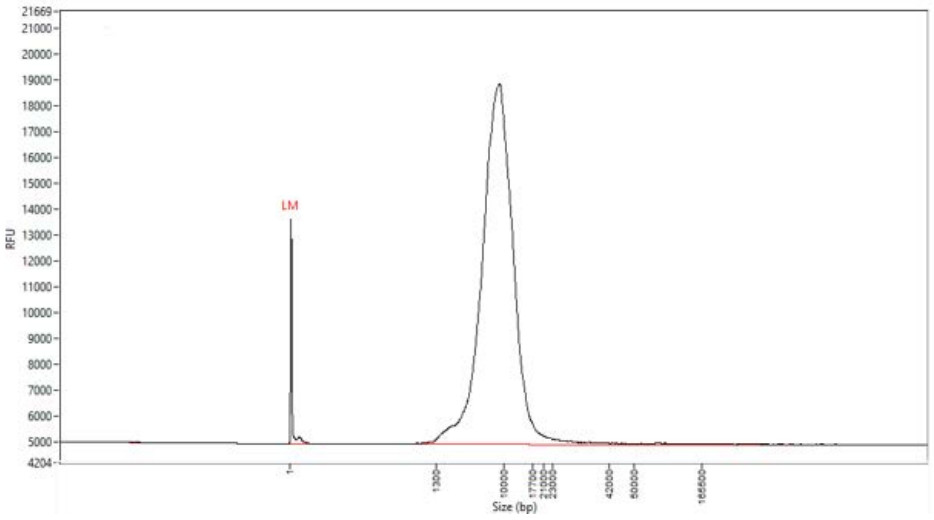

Figure 2 gDNA sample sheared using a Covaris g-TUBE and analyzed using Agilent Femto Pulse system with a Genomic DNA 165 kb Kit.

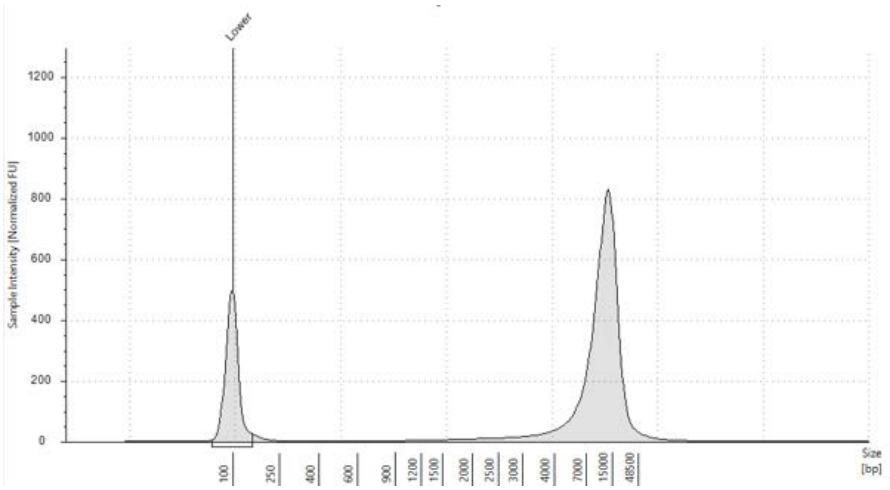

Figure 3 gDNA sample sheared using a Covaris g-TUBE and analyzed using Agilent TapeStation system with a Genomic DNA ScreenTape assay.

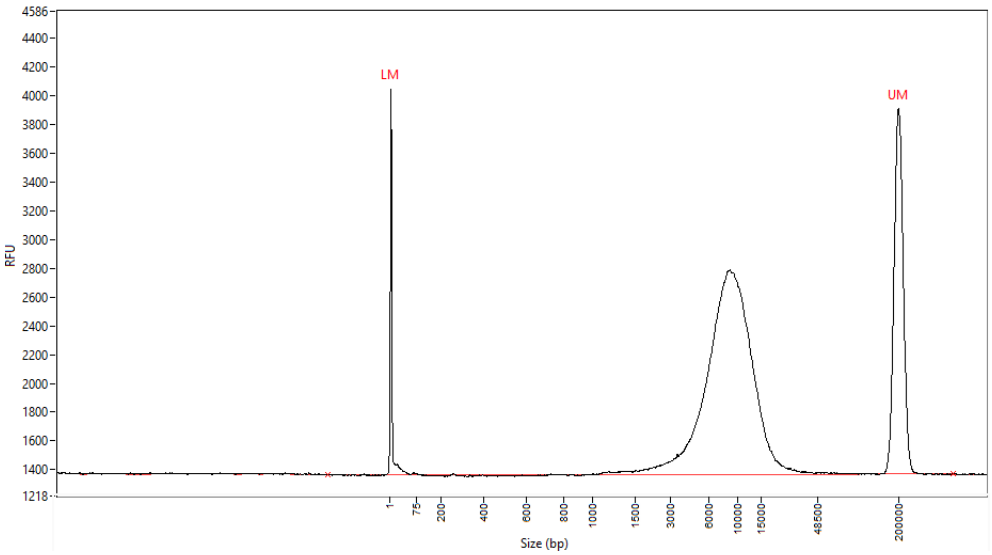

Figure 4 gDNA sample sheared using a Covaris g-TUBE and analyzed using Agilent Fragment Analyzer system with a HS Large Fragment 50 kb Kit.

Segment 2: Prepare Long-fragment SureSelect Max DNA Libraries

An abbreviated library preparation protocol is provided below. See the published protocol if additional details are needed. Modifications to the published protocol recommended for long-fragment library preparation are marked with arrows and bold text.

Step	Summary of Conditions
2.1 Prepare Ligation master mix	Per 8 reactions: 207 µL Ligation Buffer + 18 µL T4 DNA Ligase Prepare at room temperature (RT)> mix> spin> keep at RT 30–45 min before use.
2.2 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 Prepare on ice> mix> spin> keep on ice.
2.3 End-Repair and dA-Tail the DNA fragments	50 µL sheared DNA + 20 µL End Repair/dA-Tailing master mix Mix by pipetting > spin> incubate in thermal cycler: 15 min @ 20°C, 15 min @ 65°C, Hold @ 4°C.
2.4 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.
2.5 Prepare fresh 70% ethanol	Prepare a fresh 70% ethanol dilution for the purification bead washes in steps 2.6, 2.8 and 2.10. Prepare 800 µL of 70% ethanol per sample, plus excess. The freshly-prepared 70% ethanol may be used for all purification steps run on the same day. For an 8-sample run, prepare ~15 mL to cover the full workflow.
Modified 2.6 Purify adaptor-ligated DNA	100 µL DNA sample + 80 µL SureSelect Max purification bead suspension Mix by pipetting > **incubate 20 min**> collect beads> discard supernatant. Wash beads 2X with 200 µL 70% ethanol. Elute DNA in 51 µL nuclease-free H_2O> mix> **incubate 10 min**> collect beads> transfer 50 µL supernatant to fresh well.
Modified 2.7 Prepare reagents for size selection in step 2.8	**1** Dilute SureSelect Max Purification Beads to make a 35% suspension using 10 mM Tris, pH8 at RT. Mix beads until homogeneous and consistent in color before removing the aliquot for dilution and after combining with the Tris solution. Take care that no liquid droplets stick to the pipet tip when pipetting the purification beads. Incomplete transfer of the purification beads can impact results. Prepare enough for 170 µL diluted beads per sample, plus excess. For an 8 sample run, prepare 1.5 mL by combining 525 µL bead suspension + 975 µL 10 mM Tris, pH8.

Step	Summary of Conditions
Modified 2.8 Size-select sheared DNA long fragments	**1** Add 170 µL of the **35% SureSelect Max Purification Bead dilution** prepared in step 2.7 to each 50 µL sheared DNA sample. Immediately mix by pipetting up and down 15–20 times. **2** Incubate at room temperature for 20 minutes. **3** Put the DNA sample strip tube into a magnetic separation device. Wait for the solution to clear (approximately 2 to 5 minutes). **4** Keep the strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well. **5** Continue to keep the strip tube in the magnetic stand while you dispense 200 µL of fresh 70% ethanol in each sample well. **6** Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol. **7** Repeat step 5 and step 6 once for a total of two washes. **8** 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. **9** Dry the samples by keeping the unsealed strip tube at room temperature for 2 minutes or until the residual ethanol has just evaporated. 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. **10** Elute the library by adding 51 µL of nuclease-free water to each sample well. **11** Mix by pipetting up and down 10–15 times, being careful not to pellet the beads. **12** Incubate for 10 minutes at room temperature. **13** Put the strip tube in the magnetic stand and leave until the solution is clear (up to 5 minutes). **14** Transfer the cleared supernatant (approximately 50 µL) to a fresh well. Keep on ice. You can discard the beads at this time.
2.9 Index and amplify library	20 µL DNA sample + 25 µL Amplification Master Mix + 5 µL SureSelect Max UDI Primers for ILM Mix by pipetting> spin> amplify in thermal cycler using program in Table 6. Note the modified cycling conditions.
Modified 2.10 Purify indexed library DNA	50 µL amplified DNA + **25 µL** purification bead suspension Mix> **incubate 20 min**> collect beads> discard supernatant. Wash beads 2X with 200 µL 70% ethanol. Elute DNA in 15 µL nuclease-free H_2O> mix> **incubate 10 min**> collect beads> transfer 14 µL supernatant to fresh well.
2.11 Quantify and qualify DNA	Analyze quantity and quality as detailed in "Pre-capture long-read library QC.".

Modified **Table 6** Library indexing/amplification thermal cycler program for long-read libraries (50 µL vol; heated lid at 105°C)

Segment	Number of Cycles	Temperature	Time
1	1	**96°C**	**3 minutes**
2	**8**	**98°C**	**30 seconds**
		60°C	30 seconds
		68°C	**7.5 minutes**
3	1	**68°C**	**3 minutes**
4	1	4°C	Hold

Pre-capture long-read library QC

Analyze an aliquot of the prepared library DNA using one of the platforms listed in Table 4. Follow the instructions in the linked user guide. **Pre-capture libraries typically require dilution (~4X) before analysis to ensure that the DNA concentration is in the linear range of the assay**.

Table 7 Pre-capture library analysis options

Analysis platform	Assay used at this step	Link to assay instructions	Amount of sample to analyze
Agilent Femto Pulse system	Genomic DNA 165 kb Kit	Genomic DNA 165 kb Kit Quick Guide for the Femto Pulse	2 µL of diluted sample
Agilent 4200/4150 TapeStation systems	Genomic DNA ScreenTape	Genomic DNA ScreenTape Assay Quick Guide	1 µL of diluted sample
Agilent 5200/5300/5400 Fragment Analyzer systems	HS Large Fragment 50 kb Kit	HS Large Fragment 50 kb Kit Quick Guide	2 µL of diluted sample

Representative Femto Pulse, TapeStation, and Fragment Analyzer electropherograms are provided in Figure 5, Figure 6, and Figure 7 to illustrate typical results. Using the 0.5 kb to 60 kb region of the electropherogram, determine the average fragment size and the library concentration. See Table 8 for fragment size distribution guidelines.

Table 8 Expected pre-capture library fragment size guidelines

Sample analyzed	Expected average fragment size (0.5–60 kb region)
Pre-capture long-read libraries	7 to 12 kb

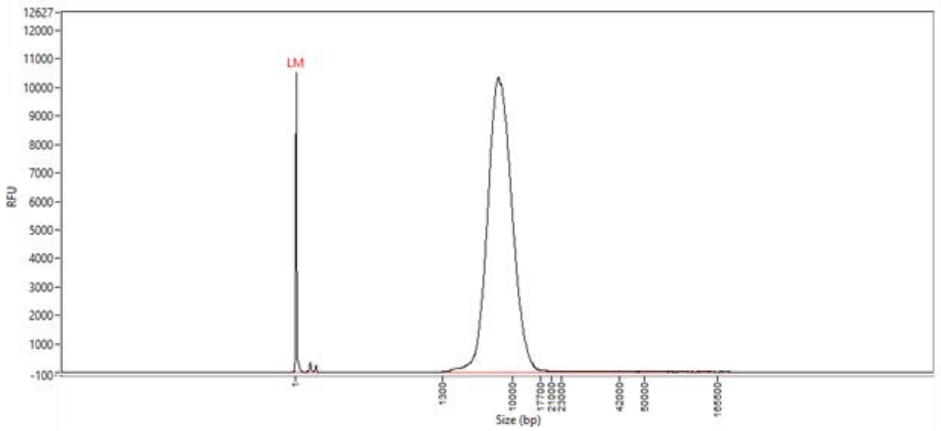

Figure 5 Pre-capture long-read library sample analyzed using Agilent Femto Pulse system with a Genomic DNA 165 kb Kit.

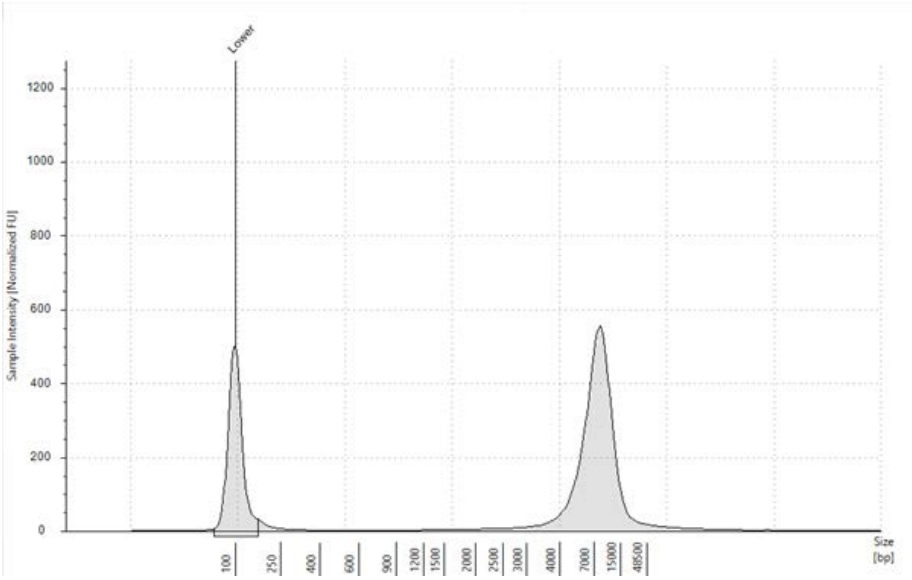

Figure 6 Pre-capture long-read library sample analyzed using Agilent TapeStation system with a Genomic DNA ScreenTape assay.

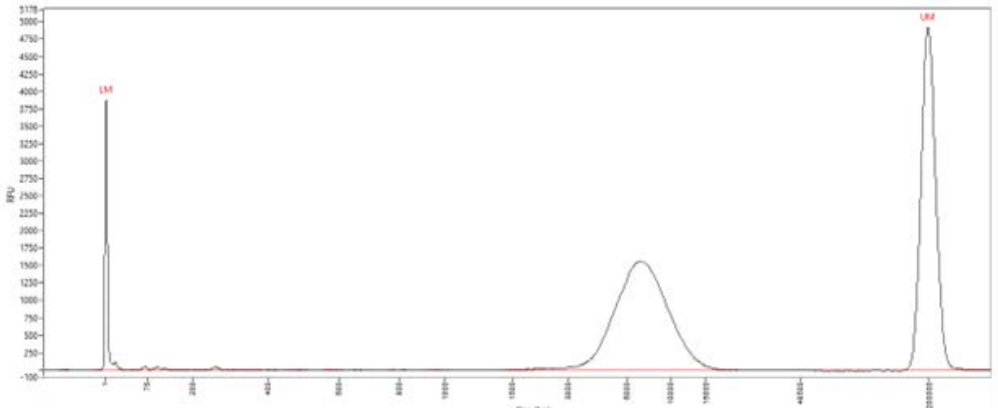

Figure 7 Pre-capture long-read library sample analyzed using Agilent Fragment Analyzer system with a HS Large Fragment 50 kb Kit.

Segment 3: Target-enrich the Long-fragment Libraries

An abbreviated fast-hyb target enrichment protocol is provided below. See the published protocol for full reagent handling and sample processing details. Modifications to the published protocol for long-fragment libraries are marked with arrows and bold text.

Step 3.1. Hybridize libraries to probe

Substep	Summary of Conditions
3.1.1 Place libraries in hyb wells	Place 12 µL of each prepared library (post-cap pooling workflow) or library pool (pre-cap pooling workflow) in nuclease-free H_2O in separate strip tube well> keep on ice. Post-cap pooling workflow: requires ≥500 ng of library in 12 µL volume Pre-cap pooling workflow: requires 500 ng of each library in 12 µL total volume
3.1.2 Program thermal cycler	Input the appropriate hybridization program in Table 9. Note the modified pre-blocking temperature. Pre-warm before loading samples.
3.1.3 Run pre-hybridization blocking protocol	Add 5 µL Blocker Mix ILM to each sample well; mix> spin> place strip in cycler. Run paused thermal cycler program segments 1 through 3; prepare Probe/Hyb Mix while program runs.
3.1.4 Prepare Probe/Hyb Mix	Prepare the Probe/Hyb Mix for your probe design size--see Table 10. Prepare at RT> mix by pipetting> spin> keep briefly at RT.
3.1.5 Run the hybridization	With samples held in cycler (Segment 3 Hold), add 13 µL Probe/Hyb Mix to wells> mix by pipetting. Seal wells completely> mix by pipetting> spin briefly> return strip to cycler. Resume cycler program, completing the remaining hybridization segment(s) and final 68°C Hold. Prep capture reagents during hybridization (see Step 3.2. Capture hybridized libraries on page 19)

Table 9 Hybridization program (30 µL vol; heated lid at 105°C)

Segment	Probe	Cycles	Temperature (Purpose)	Time
1	All probes	1	95°C (Pre-blocking)	**45 seconds**
2	All probes	1	65°C (Blocking)	10 minutes
3	All probes	1	65°C (Hyb reagent addition hold)	Hold
Modified 4	All probes	60	69°C (Hyb cycles)	1 minute
			37°C (Hyb cycles)	3 seconds
5	SureSelect XT HS Human All Exon V7/V8/V8+UTR/V8+NCV or Clinical Research Exome V4	1	69°C (Extended hyb incubation-include for listed probes only)	60 minutes
			69°C (Hold for capture start)	Hold *briefly*
	All other XT HS probes	1	69°C (Hold for capture start)	Hold *briefly*

Table 10 Preparation of Probe/Hyb Mix

Reagent	Probe designs ≥3 Mb		Probe designs<3 Mb	
	Per Hyb Reaction	8 Hybs	Per Hyb Reaction	8 Hybs
Nuclease-free water	1.5 µL	13.5 µL	4.5 µL	40.5 µL
SureSelect RNase Block (purple cap)	0.5 µL	4.5 µL	0.5 µL	4.5 µL
SureSelect Max Fast Hyb Buffer	6 µL	54 µL	6 µL	54 µL
Probe	5 µL	45 µL	2 µL	18 µL
Total	13 µL	117 µL	13 µL	117 µL

Step 3.2. Capture hybridized libraries

Substep	Summary of Conditions
3.2.1 Aliquot Wash Buffer 2	Aliquot 6 × 200 µL of SureSelect Wash Buffer 2 for each sample.
3.2.2 Prepare streptavidin beads	For each sample, wash 50 µL SureSelect Streptavidin beads 3× in 200 µL SureSelect Binding Buffer. After final wash, resuspend beads in 200 µL SureSelect Binding Buffer. Pre-warm beads/buffer in thermal cycler at 69°C for last 10 minutes of hybridization.
Modified 3.2.3 Capture hybridized libraries on streptavidin beads	Add hybridized samples (~30 µL) to pre-warmed beads (200 µL) held in cycler> mix well by pipetting. Incubate capture strip in thermal cycler at 69°C for **5 minutes**. During incubation, pre-warm the 6 × 200 µL aliquots of SureSelect Wash Buffer 2 to 70°C.
3.2.4 Wash captured libraries	Spin capture strip briefly> collect beads using a magnetic stand> discard supernatant. Wash beads 1× with 200 µL SureSelect Wash Buffer 1 at RT. Wash beads 6× with 200 µL pre-warmed SureSelect Wash Buffer 2 (for each wash: add 200 µL 70°C Wash Buffer 2> mix well by pipetting at RT> incubate 5 minutes at 70°C> collect beads at RT> discard supernatant). Resuspend washed beads in 24 µL nuclease-free H_2O> pipette to mix> keep on ice.

Step 3.3. PCR-amplify, clean up, and QC captured libraries

Substep	Summary of Conditions
3.3.1 Program thermal cycler	Input the appropriate thermal cycler program in Table 11. Note the modified cycling conditions. Pre-warm before loading samples.
3.3.2 Prepare Post-capture PCR Mix	Per 8 Hyb reactions: 225 µL Amplification Master Mix + 9 µL SureSelect Post-Capture Primer Mix Prepare on ice> mix> spin> keep on ice.
3.3.3 Amplify the bead-bound captured libraries	Add 26 µL Post-capture PCR Mix to each well containing 24 µL captured library bead suspension; pipette to mix (do not spin)> place strip in cycler> run thermal cycler program.

Substep	Summary of Conditions
Modified 3.3.4 Purify amplified libraries	50 µL amplified library bead suspension + **25 µL** purification bead suspension> mix> **incubate 20 min**> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute in 25 µL Low TE Buffer> mix> **incubate 10 min**> collect beads> retain supernatant.
3.3.5 Quantify and qualify libraries	Analyze quantity and quality as detailed on page 20.

Modified

Table 11 Post-Capture PCR thermal cycler program (50 µL vol; heated lid at 105°C)

Segment	Number of Cycles	Temperature	Time
1	1	**96°C**	**3 minutes**
2	**Probes <0.2 Mb: 17–20 cycles** **Probes 0.2–3 Mb: 15–19 cycles** **Probes 3–5 Mb: 14–15 cycles** **Probes >5 Mb: 13–14 cycles**	**98°C**	**30 seconds**
		60°C	30 seconds
		68°C	**7.5 minutes**
3	1	**68°C**	**3 minutes**
4	1	4°C	Hold

Post-capture long-read library QC

Analyze an aliquot of the sheared DNA using one of the platforms listed in Table 4. Follow the instructions in the linked user guide. P**ost-capture libraries typically require dilution (~3X) before analysis to ensure that the DNA concentration is in the linear range of the assay**.

Table 12 Post-capture library analysis options

Analysis platform	Assay used at this step	Link to assay instructions	Amount of sample to analyze
Agilent 4200/4150 TapeStation systems	Genomic DNA ScreenTape	Genomic DNA ScreenTape Assay Quick Guide	1 µL of diluted sample
Agilent Femto Pulse system	Genomic DNA 165 kb Kit	Genomic DNA 165 kb Kit Quick Guide for the Femto Pulse	2 µL of diluted sample
Agilent 5200/5300/5400 Fragment Analyzer systems	HS Large Fragment 50 kb Kit	HS Large Fragment 50 kb Kit Quick Guide	2 µL of diluted sample

Representative Femto Pulse, TapeStation, and Fragment Analyzer electropherograms are provided inFigure 8, Figure 9, and Figure 10 to illustrate typical results.

Using the 0.5 kb to 60 kb region of the electropherogram, determine the average fragment size and the library concentration. See Table 13 for fragment size distribution guidelines.

Table 13 Expected post-capture library fragment size guidelines

Sample analyzed	Expected average fragment size (0.5–60 kb region)
Post-capture long-read libraries	5 to 9 kb

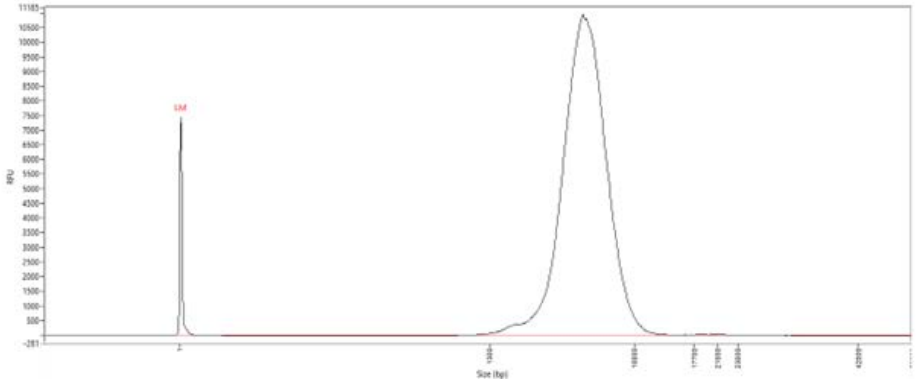

Figure 8 Post-capture long-read library sample analyzed using Agilent Femto Pulse system with a Genomic DNA 165 kb Kit.

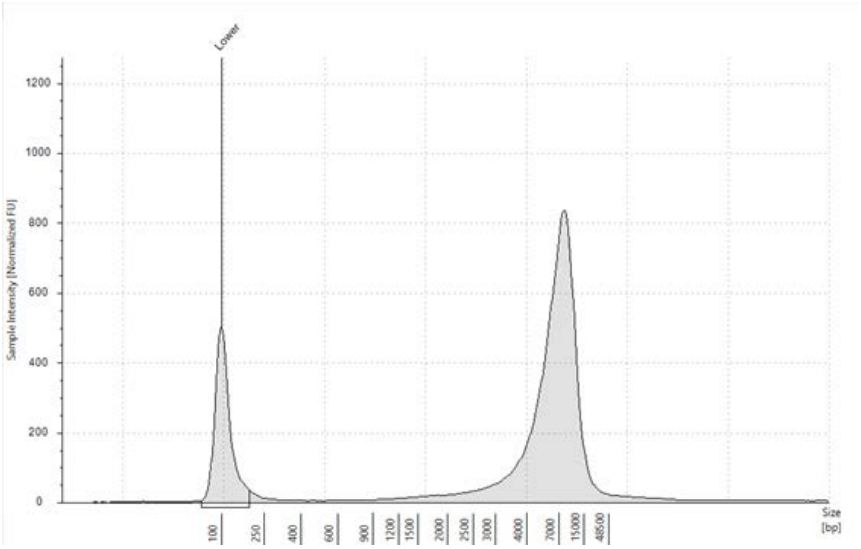

Figure 9 Post-capture long-read library sample analyzed using Agilent TapeStation system with a Genomic DNA ScreenTape assay.

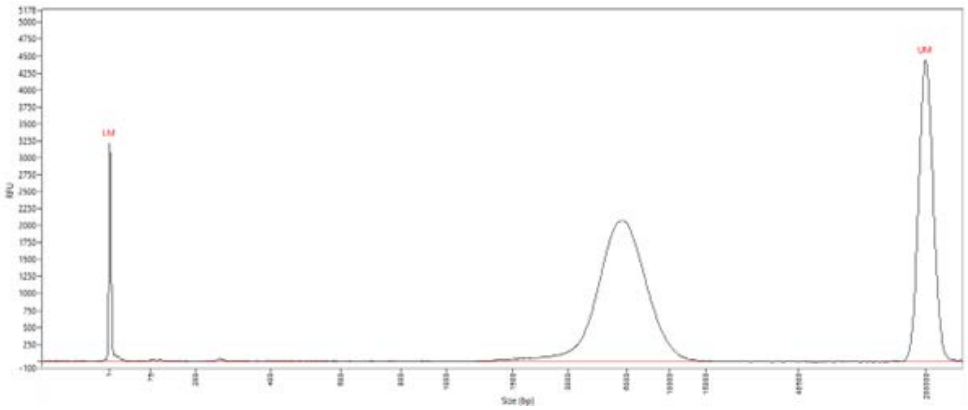

Figure 10 Post-capture long-read library sample analyzed using Agilent Fragment Analyzer system with a HS Large Fragment 50 kb Kit.

Segment 4: Convert Libraries for Long-read NGS

Before you begin this step, review the protocols provided by your selected long-read NGS provider (see Table 14) and make sure your laboratory is equipped with the additional reagents and equipment specified in the conversion and sequencing protocol.

Table 14 Long-read NGS system protocol links

Long-read NGS supplier	Conversion protocol link
Oxford Nanopore Technologies (ONT)	Ligation Sequencing Amplicons – Native Barcoding Kit 24 Ligation Sequencing DNA V14 –Ligation Sequencing Kit*
Pacific Biosciences (PacBio)	Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0

* Agilent testing was performed using the Native Barcoding Kit 24. Agilent has not tested the Ligation Sequencing Kit in the long-read NGS workflow for ONT.

Conversion for ONT long-read sequencing

The conversion protocol uses 200 fmole of each SureSelect Max library with a separate conversion reaction set up for each indexed SureSelect Max library. Libraries are pooled during the ONT conversion protocol after barcode ligation.

Complete the conversion protocol using the manufacturer's instructions. Analyze the final converted libraries as directed by ONT.

The converted libraries are sequenced on ONT MinION, PromethION, or GridION devices as described in the linked user guide.

Conversion for PacBio long-read sequencing

Indexed SureSelect Max libraries should be pooled prior to the PacBio conversion steps, using a total library pool DNA amount that satisfies the PacBio DNA input mass requirements. Refer to the protocol link in Table 14 to determine the required input mass for your PacBio platform and probe panel size. When the indexed libraries are similar in average fragment size, it is acceptable to pool by mass. When average fragment sizes differ between libraries, pool by molarity to ensure even coverage.

Complete the conversion protocol using the manufacturer's instructions. Analyze the final converted library pool as directed by PacBio.

The converted libraries are sequenced on PacBio Revio or Vega sequencers as described in the linked user guide.

3
Reference Information

This section contains reference information including information on purification bead substitution, a summary of pre-conversion library fragment composition, and troubleshooting suggestions.

Substitution of AMPure XP beads for SureSelect Max Purification Beads

AMPure XP beads have similar performance characteristics to SureSelect Max Purification Beads in the protocols provided in this guide. The beads are not, however, identical in composition and the average fragment length and yield results may differ for these two bead sources.

When using AMPure XP beads, the size selection step (page 15) should be performed using an adjusted optimal bead:DNA sample volume ratio. The modified size selection protocol parameters are summarized in Table 15. Otherwise, follow the protocol as described on page 15.

Table 15 AMPure XP bead size-selection protocol parameters

Parameter	Value
AMPure XP bead dilution conditions	Dilute beads to 35% using 10 mM Tris, pH8 at RT
Volume of 35% bead suspension added to each sample well	**175 µL** (replaces 170 µL SureSelect Max Purification Beads)
Final elution solvent and volume	50 µL nuclease-free water
Amount of eluted sample transferred to fresh well	Approximately 50 µL

The remaining bead purification steps can be performed using AMPure XP beads with no protocol changes (see page 14 and page 20). Note that while both bead types have the same optimal bead:DNA sample volume ratio in these steps, the average fragment length and yield results may differ.

Pre-conversion SureSelect Max Library Composition

The SureSelect Max library fragments prepared using the SureSelect Max reagent modules referenced in this publication are shown in Figure 11. Each fragment in the prepared library contains one target insert surrounded by sequence motifs used for multiplexed sequencing. The libraries are ready for conversion to long-read sequencing libraries using ONT or PacBio conversion systems.

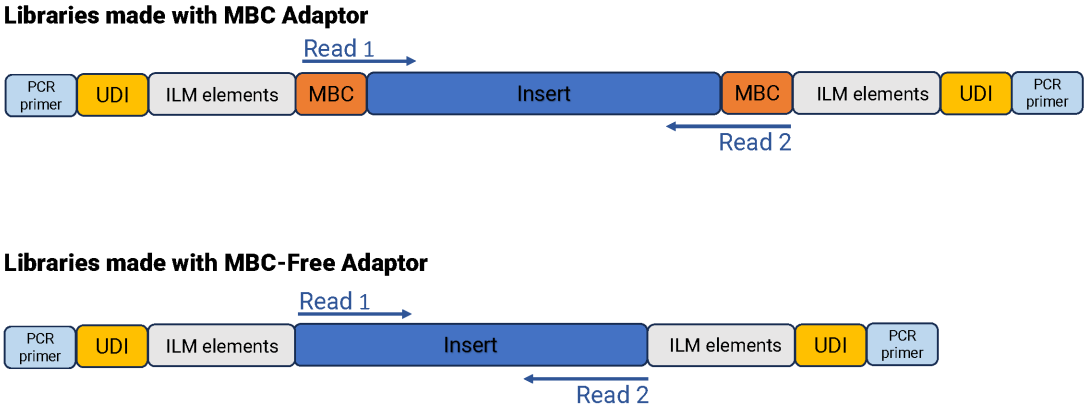

Figure 11 Content of SureSelect Max sequencing libraries. Each fragment contains one target insert (dark blue) surrounded by paired-end Illumina sequencing elements (gray), unique 8-bp dual indexes (gold), library PCR primers (light blue) and optional molecular barcode (MBC) sequences. MBCs include a 3-bp degenerate barcode plus dark bases. Index sequences can be obtained by downloading the SureSelect Max Index Sequence Resource Excel spreadsheet from Agilent.com.

Data Analysis Guidance

For PacBio systems, Agilent recommends using lima to trim the adapter sequences. Trimming needs to remove both the Illumina elements and the PacBio elements.

For ONT systems, Agilent recommends performing single- or dual-end demultiplexing in Dorado with the no-trim flag. Then, run Cutadapt with the Dorado output to trim the adapter sequences. Trimming needs to remove both the Illumina elements and the ONT elements.

For both systems, you can align trimmed reads to the reference genome using Minimap.

Index sequences for SureSelect Max libraries can also be obtained from the Agilent website. Download the SureSelect Max Index Sequence Resource Excel spreadsheet.

Demultiplexing for PacBio Systems

Before starting a run, create the necessary files as described below.

- Download the fasta file **Template_SSELUDIadaptors** from the Agilent website (https://download.chem.agilent.com/ngs/Template_SSELUDIadaptors.fasta).Use this file as a template to create a fasta file containing the SureSelect Max index sequences used in your library. Import the completed fasta file into SMRT Link via Data Management.
- Go to https://www.pacb.com/multiplexing and download the barcoded sample name file **Ampli-Fi_Barcoded_Sample_Name_file.csv**. Use this file as a template to assign sample names for each run.

Follow the guidelines below to demultiplex the reads based on the SureSelect Max index sequences and remove PCR duplicate reads.

- For MBC-tagged libraries:
  - Use the lima command tool for demultiplexing. In the output bam file, change the suffix of the file from *bam* to *fastq.gz*.
  - Use Cutadapt to trim the 5-bp MBC from both the 5' and 3' ends in the fastq file using command **cutadapt -u 5 -u -5**.
- For MBC-free libraries:
  - In the run design settings, select **NO** in the **Sample is indexed** field to indicate that the libraries were not barcoded using the SMRTbell index.
  - After sequencing, complete the following steps in SMRT Link:
    a Run the SMRT Analysis **Demultiplex Barcodes** data utility job.
    b In the Barcode Set drop-down list, select the fasta file containing the SureSelect Max index sequences.
    c For the setting **Same Barcodes on Both Ends of Sequence**, select **No**.
    d Under the Advanced Analysis Parameters menu, add **--neighbors** to the Advanced lima Options text box.
- Run the **Mark PCR Duplicates** data utility job to remove PCR duplicate reads or use the pbmarkdups command line tool (pbbioconda).

  Additional memory may be required to process the Ampli-Fi samples using the Advanced Parameters. Recommended additional task memory is 16,000 MB.

Demultiplexing for ONT Systems

Download the toml file and fasta file

Before starting a run, download the *agilent_hyb.toml* file and the *agilent_hyb.fasta* file from the Agilent website.

agilent_hyb.toml: https://download.chem.agilent.com/ngs/agilent_hyb.toml

agilent_hyb.fasta: https://download.chem.agilent.com/ngs/agilent_hyb.fasta

The toml file defines the index sequencing for the SureSelect Max DNA libraries. The fasta file is used to trim Illumina elements.

Download and install Dorado

Download the latest Dorado release from: https://github.com/nanoporetech/dorado

Example (v1.3.1 for Linux x64):

wget https://cdn.oxfordnanoportal.com/software/analysis/dorado-1.3.1-linux-x64.tar.gz

Extract the archive:

tar -xvzf dorado-1.3.1-linux-x64.tar.gz

Run demultiplexing

Follow the guidelines below to perform demultiplexing in Dorado.

NOTE

- Make sure *agilent_hyb.toml* and *agilent_hyb.fasta* are present in your working directory or provide full file paths.
- Adjust *-t* to match the number of available CPU threads.
- Replace *basecalled.bam* with the path to your input BAM file.

- Basic Demultiplexing Command:

```
./dorado-1.3.1-linux-x64/bin/dorado demux \
  --barcode-arrangement agilent_hyb.toml \
  --kit-name SSEL_Max_Pair_Custom \
  --barcode-sequences agilent_hyb.fasta \
  -t 6 \
  --output-dir demux_8plex_singleend \
  basecalled.bam
```

- Optional Parameters

  Require barcodes at both ends of reads:

  --barcode-both-ends

Example including optional flag:

```
./dorado-1.3.1-linux-x64/bin/dorado demux \
 --barcode-arrangement agilent_hyb.toml \
 --kit-name SSEL_Max_Pair_Custom \
 --barcode-sequences agilent_hyb.fasta \
 --barcode-both-ends \
 -t 6 \
 --output-dir demux_8plex_bothends \
 basecalled.bam
```

Trim and remove duplicates

Use Cutadapt to trim the 5-bp MBC from both the 5' and 3' ends in the fastq file using command **cutadapt -u 5 -u -5**.

Follow the guidelines below to remove duplicate reads.

- To mark duplicates with Picard, open a terminal window and run the following command:

  ```
  java -jar /usr/picard/picard.jar MarkDuplicates READ_NAME_REGEX=null
  OPTICAL_DUPLICATE_PIXEL_DISTANCE=0 TAGGING_POLICY=All
  REMOVE_DUPLICATES=false METRICS_FILE=$(BAM.%bam).duplicate_metrics.txt
  INPUT=$BAM OUTPUT=${BAM%.bam}.markdup.bam
  ```

- To remove duplicates with samtools, open a terminal window and run the following command:

  ```
  samtools rmdup -s --output-fmt BAM --reference $GENOME ${bam}
  ${bam%.bam}.rmdup.bam
  ```

Troubleshooting Guide

If <600 ng input gDNA available

The standard protocol calls for shearing 600 ng input gDNA in a 150 µL sample volume. These shearing sample conditions are determined by the 150 µL shearing volume in the Covaris g-TUBE protocol and the library preparation sample conditions of 200 ng sheared DNA per 50 µL sample volume.

✓When 600 ng gDNA is not available, shearing may be performed using 200 ng gDNA in a 50 µL sample volume, with minimal impacts on performance expected.

- Agilent observes similar fragment size profiles and long-read library preparation results for samples sheared in 50 µL or 150 µL volumes, while following the other g-TUBE protocol conditions recommended by Covaris.
- Before adopting this protocol modification, performance should be verified for your centrifuge and operating conditions. Optimization of centrifugation speed and duration may be required.

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.

✓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. Monitor the bead pellets frequently while drying and conclude the drying step immediately after the residual ethanol has evaporated.

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

✓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 each 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. Take care that no liquid droplets stick to the pipet tip when pipetting the purification beads. Incomplete transfer of the purification beads can impact results. Evaporation of the bead storage buffer can occur when the bottle of beads has been repeatedly opened. If buffer evaporation is suspected, try a fresh bottle of beads.

✓The average library fragment size can be increased by decreasing the ratio of bead suspension to DNA sample volume in the initial fragment size selection procedure.

The provided size selection protocol uses 170 µL µL of 35% SureSelect Max Purification Bead suspension added to each 50 µL sample. To increase the average fragment size in the library, add 165 µL µL of 35% bead suspension to each 50 µL sample.

Note that using this protocol modification designed to remove additional short DNA fragments will also decrease the overall library yield.

If post-capture QC electropherogram includes low molecular weight (<3 kb) shoulder

✓Excess low molecular weight fragments in the library, evidenced by a <3 kb shoulder, can be removed by performing an additional round of size-selection just before library conversion using the procedure below:

1. Make a 35% (v/v) dilution of SureSelect Max Purification Beads in 10 mM Tris, pH8 at room temperature. Prepare enough for 93 µL diluted beads per sample, plus excess.
2. Add 92.5 µL (3.7X) of the 35% SureSelect Max Purification Bead suspension to each 25 µL target-enriched library sample that requires size-selection. Mix thoroughly until the beads are evenly distributed.
3. Incubate at room temperature for 20 minutes.

4 Put the DNA sample strip tube into a magnetic separation device. Wait for the solution to clear (approximately 2 to 5 minutes).

5 Keep the strip tube in the magnetic stand. Carefully remove and discard the cleared solution from each well without disturbing the beads.

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

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

8 Repeat step 6 and step 7 once for a total of two washes.

9 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.

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

11 Elute the library by adding 26 µL of Low TE Buffer.

12 Mix thoroughly until the beads are evenly distributed.

13 Incubate for 5 minutes at room temperature.

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

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

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In This Guide

This technical guide provides recommendations for adapting Agilent SureSelect Max DNA library preparation and target enrichment protocols to a long-read NGS workflow.

Version A0, February 2026

G9963-90001

Agilent