SureSelect Max Target Enrichment using Overnight Hybridization

For NGS using the Illumina Platform

Protocol

Version A0 September 2024

SureSelect platform manufactured with Agilent SurePrint technology.

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

Notices

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Acknowledgment

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In this Guide...

This guide provides an optimized protocol for target enrichment of Illumina paired-end multiplexed DNA or cDNA sequencing libraries using SureSelect Max Overnight Hybridization Modules and a SureSelect probe. Libraries can be pooled for NGS using either pre-capture or post-capture pooling. The target-enriched libraries are ready for NGS using a suitable Illumina instrument. Before starting this protocol, SureSelect Max DNA or RNA libraries must be prepared as described in separate guides for earlier workflow segments.

Content

1 Before You Begin

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

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

Overview of the Workflow

The SureSelect Max system offers flexible workflow options for preparation and target enrichment of DNA or RNA libraries for NGS, with kits for specific workflow segments in a modular format. This publication provides optimized protocols for the target enrichment workflow segment, using overnight hybridization conditions for target enrichment of prepared SureSelect Max DNA or RNA libraries with a compatible SureSelect XT probe, summarized in Figure 1. The overnight hybridization protocol includes options for post-capture or pre-capture library pooling.

For detailed protocols see "Target Enrichment with Overnight Hybridization Protocol" on page 10. Protocols for the upstream DNA or RNA library preparation modules are provided in separate publications.

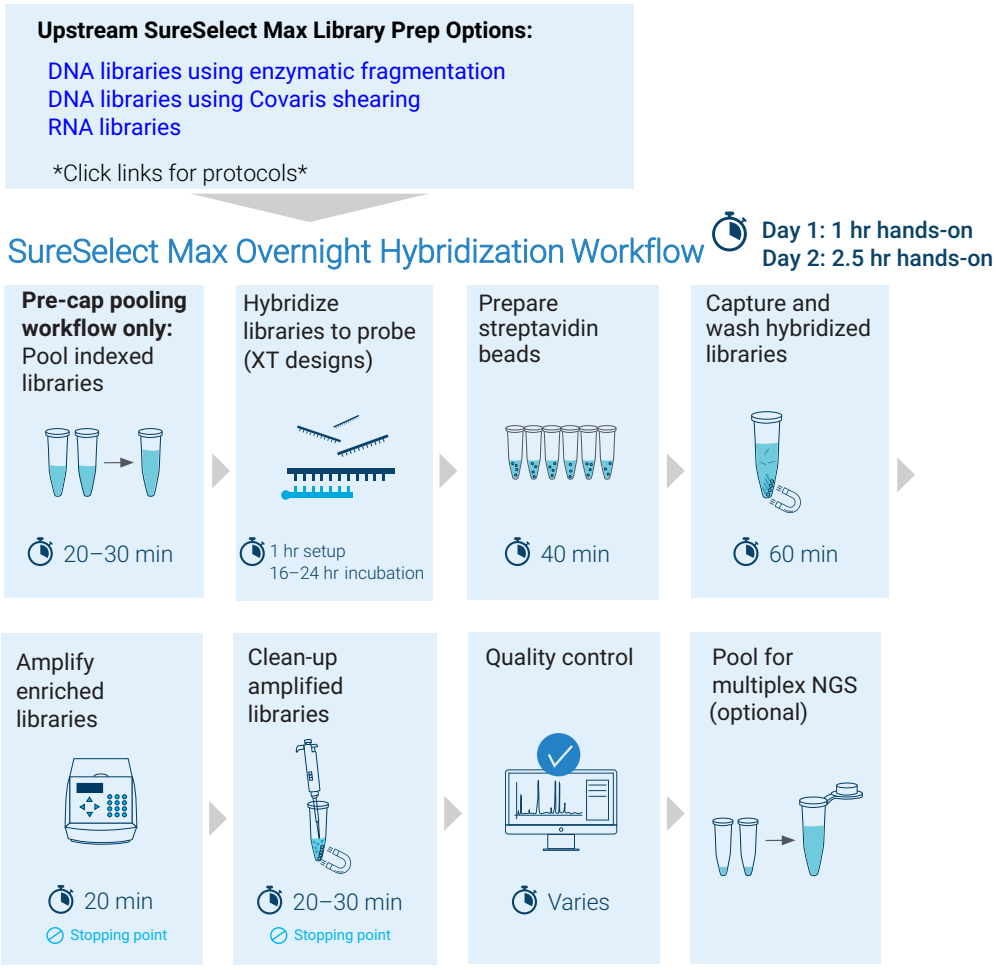

Figure 1 Summary of the SureSelect Max Overnight Hyb target enrichment workflow segment, including optional stopping points and estimated time requirements. Estimates are guidelines for 16 reaction runs. Timing for runs using different protocol parameters may vary.

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

SureSelect Max Modules and Probes Used in the Workflow

This publication provides optimized protocols for the target enrichment workflow segment, using overnight hybridization conditions for target enrichment of prepared SureSelect Max DNA or RNA libraries with a SureSelect XT probe. Agilent's SureSelect reagents required to complete the protocols are summarized in Table 1 and Table 2.

Table 1 SureSelect Max kits used in the Target Enrichment with Overnight Hybridization workflow

Kit Description	16 Hybridization Kits*	96 Hybridization Kits
SureSelect Max Overnight Hyb Kit†	G9690A	G9690B
SureSelect Max Blockers and Primers Kit for ILM	G9699A	G9699B
SureSelect Max Purification Beads‡	G9962A (5 mL)	G9962B (30 mL)
SureSelect XT Probe	See Table 2 for ordering information. Select Overnight Hyb/XT design probes for use in the SureSelect Max Overnight Hybridization workflow. Select the appropriate formulation and format for your indexed library pooling workflow choice (post-capture or pre-capture pooling).	

* 16 Hyb Kits can be used to process 16 prepared libraries in a post-capture pooling workflow, or up to 96 prepared libraries in a pre-capture pooling workflow.

† Includes SureSelect Streptavidin Beads. Separate purchase not required.

‡ May be substituted with AMPure XP beads (see Table 3 on page 8).

Table 2 Recommended Probes

Probe Description	Design ID	Ordering Information
Pre-designed Probes		
SureSelect XT Human All Exon V8	S33266436	Please visit the Agilent.com probe webpages or the SureDesign website to obtain ordering information for pre-designed probes formulated for either post-capture pooling or pre-capture pooling. Please contact Sales or your local representative if you need assistance.
SureSelect XT Human All Exon V8+UTR	S33613367	
SureSelect XT Human All Exon V8+NCV	S33700246	
SureSelect XT Human All Exon V7	S31285117	
SureSelect XT Clinical Research Exome V4	S34226363	
SureSelect XT Clinical Research Exome V2	S30409818	
Custom Probes		
SureSelect Custom Tier1 1–499 kb		Please visit the SureDesign website to design Custom SureSelect probes and obtain ordering information for custom probes formulated for either post-capture pooling or pre-capture pooling. Contact the SureSelect support team (see page 2) or your local representative if you need assistance.
SureSelect Custom Tier2 0.5 –2.9 Mb		
SureSelect Custom Tier3 3 –5.9 Mb		
SureSelect Custom Tier4 6 –11.9 Mb		
SureSelect Custom Tier5 12–24 Mb		
Agilent Community Designs: Please visit the Community Designs (NGS) webpages at agilent.com for information on custom panels developed in collaboration with experts in various fields.		Design details and ordering information are available at the SureDesign website on the *Published Designs* tab. Contact the SureSelect support team (see page 2) or your local representative if you need assistance.

Additional Materials Used in the Workflow

See Table 3 and Table 4 for additional reagents and equipment used in the workflow.

CAUTION Sample volumes exceed 0.2 mL in certain steps of this protocol. Make sure that the plasticware used with the selected thermal cycler holds ≥0.25 mL per well (see Table 3).

Table 3 Ordering Information for Additional Reagents and Equipment

Description	Vendor and Part Number	Usage Notes
Thermal Cyclers (2) with 96-well, 0.2 mL blocks	Various suppliers	Protocols require two thermal cyclers to complete a reagent pre-warming step during certain sample incubation steps. See *Note* on page 13 for more information.
Plasticware compatible with the selected thermal cycler: 8-well strip tubes Tube cap strips (flat or domed)*	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.
Vacuum concentrator	Savant SpeedVac, model DNA120, or equivalent	Used for sample dehydration prior to hybridization (4 µL sample volume required). If a vacuum concentrator is not available in your laboratory, see *Troubleshooting* on page 40 for suggested protocol modifications.
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	—
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	—
Nucleic acid analysis system	See Table 4 on page 9	—
1X Low TE Buffer	Thermo Fisher Scientific p/n 12090-015, or equivalent	10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA
Nuclease-free Water	Thermo Fisher Scientific p/n AM9930	Water should not be DEPC-treated.
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276	—
Optional: AMPure XP Kit (5 mL)	Beckman Coulter Genomics p/n A63880	Optional alternative to SureSelect Max Purification Beads (See Table 1)

* Consult the thermal cycler manufacturer's recommendations for use of either flat or domed strip caps and for any accessories (e.g., compression mats) required for optimal performance with the selected caps. Ensure that the combination of selected thermal cycler and plasticware provides complete sealing of sample wells and optimal contact between the instrument heated lid and vial cap for heat transfer.

Table 4 Recommended Nucleic Acid Analysis Platforms*

Analysis System	Vendor and Part Number Information
Agilent 4200/4150 TapeStation Instrument	Agilent p/n G2991AA/G2992AA
Consumables:	
8-well tube strips	p/n 401428
8-well tube strip caps	p/n 401425
High Sensitivity D1000 ScreenTape	p/n 5067-5584
High Sensitivity D1000 Reagents	p/n 5067-5585
Agilent 5200/5300/5400 Fragment Analyzer Instrument	Agilent p/n M5310AA/M5311AA/M5312AA
Consumables:	
HS NGS Fragment Kit (1-6000 bp)	p/n DNF-474-0500

* Libraries may also be analyzed using the Agilent 2100 Bioanalyzer instrument, p/n G2939BA, and the High Sensitivity DNA Kit (p/n 5067-4626).

Procedural and Safety Notes

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

In this workflow segment, SureSelect Max DNA or cDNA libraries are hybridized with a target-specific probe. After hybridization, the targeted molecules are captured on streptavidin beads and then PCR-amplified. The libraries can be pooled for multiplex NGS after hybridization (see page 11 for post-capture pooling workflow setup) or prior to hybridization (see page 12 for pre-capture pooling workflow setup).

CAUTION

During the overnight hybridization incubation, wells must be adequately sealed to minimize evaporation from the sample wells, or your results can be negatively impacted.

Before you do the first experiment, make sure the plasticware and capping method are appropriate for the thermal cycler. Check that no more than 4 µL is lost to evaporation under the conditions used for hybridization (see page 15).

The hybridization workflow segment uses the components listed in Table 5. Prepare as directed before use (refer to the *Where Used* column).

Table 5 Reagents for Hybridization and Capture

Storage Location	Kit Component	Preparative Steps	Where Used
–80°C	SureSelect Probe	Thaw on ice before starting Hyb setup on page 13 and keep on ice; vortex to mix	page 15
SureSelect Max Target Enrichment Kit Overnight Hyb Module Box 1, stored at RT	SureSelect Hyb 1 (orange cap or bottle)	Keep at RT, vortex to mix	page 13
	SureSelect Hyb 2 (red cap)	Keep at RT, vortex to mix	page 13
	SureSelect Hyb 4 (black cap)	Keep at RT, vortex to mix	page 13
SureSelect Max Target Enrichment Kit Overnight Hyb Module Box 2, stored at –20°C	SureSelect Hyb 3 (yellow cap)	Thaw and keep at RT, vortex to mix	page 13
	SureSelect RNase Block (purple cap)	Thaw and keep on ice, vortex to mix	page 14
SureSelect Max Blockers and Primers Module for ILM, stored at –20°C	Blocker Mix, ILM (blue cap)	Thaw and keep on ice, vortex to mix	page 14

Step 1. Place libraries or library pools in the hybridization wells

Option 1: Plate individual libraries (post-capture pooling workflow)

The hybridization reaction requires prepared library samples in 4 µL of nuclease-free water. See Table 6 for the recommended and required amounts of prepared library sample input for hybridization. To maximize complexity of the final enriched NGS library, add the full 12 µL of prepared library sample to the hybridization reaction. After plating, sample volumes are reduced to 4 µL as described below.

Agilent recommends retaining the remaining prepared library solution volume (~2 µL) for any later troubleshooting QC needs.

Table 6 Hybridization input guidelines

Type of prepared library	Recommended amount	Minimum amount*
gDNA library (DNA input)	12 µL of undiluted prepared gDNA library solution (pre-capture QC optional) reduced to 4 µL	≥500 ng library DNA
cDNA library (RNA input)	12 µL of undiluted prepared cDNA library solution (pre-capture QC optional) reduced to 4 µL	≥200 ng library cDNA

* For workflows that include the optional pre-capture QC step, use at least the minimum amount of library shown here in the hybridization reactions. When library normalization in the run is not required, using the maximum amount of each library (12 µL of undiluted sample) is still recommended to maximize complexity.

1. Prepare the hybridization strip(s) by placing the appropriate amount of each prepared library into a separate sample well.
2. Using a vacuum concentrator at ≤45°C, dehydrate the library samples to volume <4 µL without completely drying the samples. Measure the final volume in each well and then bring the volume to 4 µL with nuclease-free water.

NOTE

If a vacuum concentrator is not available in your laboratory, see *Troubleshooting* on page 40.

3 Cap the wells, then vortex the strip vigorously for 5–10 seconds. Spin briefly to collect the liquid, then keep the samples on ice until use in step 4 on page 14.

Option 2: Plate library pools (pre-capture pooling workflow)

1 Pool the indexed library samples as directed in Table 7, using equal amounts of 8 or 16 libraries, based on the probe design. For each pool, combine the appropriate volume of each prepared library sample in one well of a strip tube.

Table 7 Pre-capture pooling recommendations

Probe description	Number of indexes per pool	Library type	Total amount of library DNA per pool	Amount of each library
SureSelect XT PreCap Human All Exon and Exome probes	8	DNA	4 µg	500 ng
	8	cDNA (RNA input)	1.6 µg	200 ng
SureSelect XT PreCap Custom Probes	16	DNA	4 µg	250 ng
	16	cDNA (RNA input)	1.6 µg	100 ng

2 Using a vacuum concentrator at ≤45°C, dehydrate the prepped library pools to volume <4 µL without completely drying the samples. Measure the final volume in each well and then bring the volume of each pool to 4 µL with nuclease-free water.

NOTE

If a vacuum concentrator is not available in your laboratory, see *Troubleshooting* on page 40.

3 Cap the wells, then vortex the sample strip vigorously for 5–10 seconds. Spin briefly to collect the liquid, then keep the samples on ice until use in step 4 on page 14.

Step 2. Hybridize libraries to the SureSelect probe

NOTE

Two thermal cyclers are required for the Hybridization and Capture protocols in the following sections, to complete reagent pre-warming steps during two sample incubation steps: 1) capture bead pre-warming in step 6 on page 16 during hybridization and 2) Wash Buffer 2 pre-warming during the capture incubation (see step 2 and step 3 on page 17). If two thermal cyclers are not available in your laboratory, the reagent pre-warming steps may be completed using an alternative temperature control device. Reaction mixtures that include the library samples should be incubated using a thermal cycler where directed.

1 Prepare the Hybridization Buffer by mixing the components in Table 8 at room temperature. Keep at room temperature until use in step 7. If a precipitate forms, warm the Hybridization Buffer at 65°C for 5 minutes.

Table 8 Preparation of Hybridization Buffer

Reagent	Volume for 1 reaction	Volume for 8 Hyb reactions (includes excess)*	Volume for 24 Hyb reactions (includes excess)†
SureSelect Hyb 1 (orange cap/bottle)	6.63 µL	66.3 µL	185.6 µL
SureSelect Hyb 2 (red cap)	0.27 µL	2.7 µL	7.6 µL
SureSelect Hyb 3 (yellow cap)	2.65 µL	26.5 µL	74.2 µL
SureSelect Hyb 4 (black cap)	3.45 µL	34.5 µL	96.6 µL
Total	13 µL	130 µL	364 µL

* 16-Hyb Target Enrichment Kits contain enough reagents for 2 runs of 8 Hybs each using the indicated excess volume.

† 96-Hyb Target Enrichment Kits contain enough reagents for 4 runs of 24 Hybs each using the indicated excess volume.

2 Find the optimal blocking and hybridization temperature for your probe design in Table 9.

Table 9 Blocking and Hybridization temperature based on probe design

Probe Design	Optimal Blocking/Hybridization Temperature
SureSelect XT Human All Exon V8/V8+UTR/V8+NCV SureSelect XT Human All Exon V7 SureSelect XT PreCap Clinical Research Exome V4	67.5°C
All other XT probe designs	65°C

3 Preprogram a thermal cycler for pre-hybridization blocking as shown in Table 10. Set the heated lid to 105°C. Prewarm the instrument before samples are loaded in step 5.

Table 10 Thermal cycler program for blocking prior to hybridization

Segment	Number of Cycles	Temperature	Time
1	1	95°C	5 minutes
2	1	67.5°C or 65°C (see Table 9)	Hold (at least 5 minutes)

4 To each library sample well, add 5 µL of Blocker Mix, ILM (blue cap). Seal the wells then vortex at high speed for 5 seconds. Spin briefly to collect the liquid and release any bubbles.

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

5 Transfer the sample strip to the thermal cycler and run the program in Table 10.

Prepare the additional hybridization reagents described in step 6 and step 7 below during the blocking thermal cycler program. Make sure that the library + Blocker Mix samples are held at 67.5°C or 65°C for at least 5 minutes in Segment 2 before adding the remaining hybridization reaction components in step 8 on page 15.

6 Prepare a 25% solution of SureSelect RNase Block according to Table 11. Mix well and keep on ice.

Table 11 Preparation of RNase Block solution (includes excess)

Reagent	Volume for 1 Hyb	Volume for 8 Hybs (includes excess)	Volume for 24 Hybs (includes excess)
SureSelect RNase Block (purple cap)	0.5 µL	4.5 µL	13.0 µL
Nuclease-free water	1.5 µL	13.5 µL	39.0 µL
Total	2 µL	18 µL	52 µL

NOTE

Prepare the mixture described in step 7 below at room temperature at the time of use. Do not keep solutions containing the probe at room temperature for extended periods.

For larger run sizes, hybridization reactions can be started in step 8 below using a multichannel pipette after dividing the master mix volume prepared in step 7 into wells of a strip tube. Using this protocol modification, which includes additional pipetting losses, may require preparation of a larger reagent volume mixture.

7 Prepare the Probe Hybridization Mix appropriate for your probe design size. Use Table 12 for probes ≥3 Mb or Table 13 for probes <3 Mb. For custom probes, see the probe tube label for design size range.

Combine the listed reagents at room temperature. Mix well by vortexing at high speed for 5 seconds then spin down briefly. Keep at room temperature until added to wells in step 8.

Table 12 Preparation of Probe Hybridization Mix for **probes≥3 Mb**

Reagent	Volume for 1 Hyb	Volume for 8 Hybs (includes excess)	Volume for 24 Hybs (includes excess)
25% RNase Block solution (from step 6)	2 µL	18 µL	50 µL
Probe (with design ≥3 Mb)	5 µL	45 µL	125 µL
SureSelect Hybridization Buffer (from step 1)	13 µL	117 µL	325 µL
Total	20 µL	180 µL	500 µL

Table 13 Preparation of Probe Hybridization Mix for **probes<3 Mb**

Reagent	Volume for 1 Hyb	Volume for 8 Hybs (includes excess)	Volume for 24 Hybs (includes excess)
25% RNase Block solution (from step 6)	2 µL	18 µL	50 µL
Probe (with design<3 Mb)	2 µL	18 µL	50 µL
SureSelect Hybridization Buffer (from step 1)	13 µL	117 µL	325 µL
Nuclease-free water	3 µL	27 µL	75 µL
Total	20 µL	180 µL	500 µL

8 Keeping the library + Blocker samples in the cycler held at 67.5°C or 65°C, transfer 20 µL of the room-temperature probe hybridization mix from step 7 to each sample well.

Mix well by pipetting up and down slowly 8 to 10 times.

The hybridization reaction wells now contain approximately 29 µL.

9 Seal the wells with fresh strip caps. Make sure that all wells are completely sealed. Vortex briefly, then spin briefly to remove any bubbles. Immediately return the hybridization strip to the thermal cycler.

10 Incubate the hybridization reactions in the thermal cycler for 16 to 24 hours at 67.5°C or 65°C (see Table 9) with heated lid set to 105°C.

Step 3. Prepare streptavidin beads and buffers for capture

The capture workflow segment uses the components listed in Table 14.

Table 14 Reagents for Capture

Storage Location	Kit Component	Preparative Steps	Where Used
+4°C	SureSelect Streptavidin Beads (bottle)	Remove from 4°C just before use, vortex to mix	page 16
SureSelect Max Target Enrichment Kit Overnight Hyb Module Box 1, stored at RT	SureSelect Binding Buffer (bottle)	Ready to use	page 16
	SureSelect Wash Buffer 1 (bottle)	Ready to use	page 17
	SureSelect Wash Buffer 2 (bottle)	Ready to use	page 16

NOTE Set the thermal cycler heated lid to 105°C for all thermal cycler incubation steps used for bead preparation and library capture, including the 6X capture wash incubations at 70°C on page 17.

1. Prepare 200 µL aliquots of Wash Buffer 2 for later use in capture wash steps. Using fresh strip tubes, aliquot 6 wells of buffer for each sample in the run. Keep the aliquots at room temperature until directed to pre-warm for washes on page 17.
2. Vigorously resuspend the vial of SureSelect Streptavidin Beads on a vortex mixer. The magnetic beads settle during storage.
3. For each hybridization sample, add 50 µL of the resuspended beads to wells of a fresh PCR strip tube.
4. Equilibrate the beads in SureSelect Binding Buffer using 3× washes:
   a. Add 200 µL of SureSelect Binding Buffer.
   b. Mix by pipetting up and down 20 times or cap the wells and vortex at high speed for 5–10 seconds then spin down briefly.
   c. Put the strip tube into a magnetic separator device.
   d. Wait 5 minutes or until the solution is clear, then remove and discard the supernatant.
   e. Repeat step a through step d two more times for a total of 3 washes.
5. Resuspend the washed streptavidin beads in 200 µL of SureSelect Binding Buffer.
6. Pre-warm the streptavidin capture beads and Binding Buffer by incubating the strip tube in a thermal cycler at 68°C for 10 minutes.

Step 4. Capture the hybridized libraries

1. After the 16 to 24 hour hybridization incubation period is complete and after finishing all streptavidin bead preparation steps, add the hybridization mixture to the pre-warmed capture beads as described below.
   a. Using a multichannel pipette, transfer the entire volume (approximately 29 µL) of the hybridization mixtures to wells of pre-warmed beads, while keeping the bead strip in the thermal cycler at 68°C.

b Using a P200 pipette set to 150 µL, mix by pipetting up and down 8–10 times; make sure the beads are fully resuspended. Seal the wells with fresh caps.

2 Incubate the capture reaction strip in the thermal cycler at 68°C for 10 minutes.

3 Transfer the SureSelect Wash Buffer 2 aliquots prepared on page 16 (200 µL/well) to a thermal cycler held at 70°C. Pre-warm the buffer until used for washes in step 8.

4 When the 10 minute capture incubation (step 2) is complete, spin the samples briefly to collect the liquid.

5 Put the capture reaction strip in a magnetic separator to collect the beads. Wait until the solution is clear (approximately 1 to 2 minutes), then remove and discard the supernatant.

6 Resuspend the beads in 200 µL of SureSelect Wash Buffer 1. Mix by pipetting up and down 15–20 times, until beads are fully resuspended.

7 Return the sample strip to the magnetic separator. Wait for the solution to clear (approximately 1 minute), then remove and discard the supernatant.

Spin the samples briefly to collect any residual wash buffer and remove with a P20 pipette.

CAUTION

To ensure specificity of capture, make sure that the SureSelect Wash Buffer 2 is pre-warmed to 70°C before use in the wash steps below.

During the washes in step 8, keep the sample strip at room temperature during the pipetting and vortexing steps prior to each 70°C incubation step.

Do not use a tissue incubator, or other devices with significant temperature fluctuations, for the 70°C incubation steps.

8 **Place the capture sample strip in a rack at room temperature.** Wash the beads with Wash Buffer 2, using the steps below.

a Resuspend the beads in 200 µL of 70°C pre-warmed Wash Buffer 2. **Make sure the beads are completely in suspension before proceeding.** To ensure thorough resuspension, Agilent recommends the following two-part mixing routine. Keep the capture reactions at room temperature while mixing.

i) Using a P200 pipette set to 150 µL, pipette up and down 15–20 times, until beads are fully resuspended. Pipette slowly and gently to reduce bubble formation.

ii) Seal the wells with fresh caps and vortex at high speed for 8 seconds. Spin the strip tube briefly to collect the liquid and release any bubbles without pelleting the beads.

b Incubate the samples for 5 minutes at 70°C in a thermal cycler.

c Put the sample strip in the magnetic separator at room temperature.

d Wait 1 minute for the solution to clear, then remove and discard the supernatant. Return samples to the rack at room temperature.

e Repeat step a through step d five more times for a total of 6 washes.

When the final wash is complete, spin the samples briefly to collect any residual Wash Buffer 2 and remove with a P20 pipette.

9 Add 24 µL of nuclease-free water to each sample well. Pipette up and down 8 times to resuspend the beads.

10 Keep the samples on ice until they are used in the PCR reactions on page 19.

NOTE

The captured DNA or cDNA libraries are retained on the streptavidin beads during the post-capture amplification step.

Step 5. Amplify the captured libraries

The post-capture amplification workflow segment uses the components listed in Table 15. Prepare as directed before use (refer to the *Where Used* column).

Table 15 Reagents for post-capture amplification

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect Max Target Enrichment Kit Overnight Hyb Module Box 2, stored at –20°C	Amplification Master Mix (red cap or bottle)	Thaw on ice then keep on ice. Mix thoroughly by inversion at least 5X. **Do not vortex.**	page 19
SureSelect Max Blockers and Primers Module for ILM, stored at –20°C	SureSelect Post-Capture Primer Mix (clear cap)	Thaw and keep on ice, vortex to mix.	page 19
+4°C	SureSelect Max Purification Beads OR AMPure XP Beads	Equilibrate at room temperature for at least 30 minutes before use, vortex to mix.	page 20

1 Preprogram a thermal cycler as shown in Table 16. Set the heated lid to 105°C. Prewarm the instrument before samples are loaded in step 6 on page 19.

Table 16 Post-Capture PCR thermal cycler program (50 µL vol)

Segment	Number of Cycles	Temperature	Time
1	1	98°C	45 seconds
2	10–16 (see Table 17 for probe design size-based cycle number recommendation)	98°C	15 seconds
		60°C	30 seconds
		72°C	30 seconds
3	1	72°C	1 minute
4	1	4°C	Hold

Table 17 Post-capture PCR cycle number recommendations

Probe Design Size	Cycles	
	DNA input libraries	RNA input libraries
Probes <0.2 Mb	16 cycles	16 cycles
Probes 0.2–3 Mb	12–16 cycles	14 cycles
Probes 3–5 Mb	11–12 cycles	13 cycles
Probes >5 Mb (including Human All Exon and Exome probes)	10–11 cycles	12 cycles

NOTE To avoid cross-contamination, it is recommended to set up PCR reactions (all components except the libraries) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

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

3 Prepare the appropriate volume of post-capture PCR reaction mix as described in Table 18, on ice. Mix well on a vortex mixer.

Table 18 Preparation of post-capture PCR reaction mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 24 reactions (includes excess)
Amplification Master Mix (red cap or bottle)	25 µL	225 µL	650 µL
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	9 µL	26 µL
Total	26 µL	234 µL	676 µL

4 Add 26 µL of the PCR reaction mix prepared in Table 18 to each sample well containing 24 µL of bead-bound target-enriched library DNA or cDNA.

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

6 Place the sample strip in the thermal cycler and run the program in Table 16.

NOTE The SureSelect Max Purification Beads or AMPure XP Beads used in the next section must be equilibrated to room temperature for at least 30 minutes before use.

7 When the PCR amplification program is complete, spin the amplified library strip briefly. Proceed to purification.

NOTE In the following purification protocol, the hybrid capture streptavidin beads remain mixed with the magnetic purification beads throughout the procedure.

If preferred, you can remove the streptavidin beads prior to purification by adding the following steps: 1) Place the amplified library strip tube on the magnetic stand at room temperature 2) Wait 2 minutes for the solution to clear 3) Transfer each supernatant (approximately 50 µL) to fresh strip tube wells and proceed with the purification procedure. The streptavidin beads can be discarded after collecting the library solution.

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

Step 6. Purify the final libraries using magnetic purification beads

Purify the amplified libraries using room-temperature SureSelect Max Purification Beads or AmpPure XP Beads. Critical purification protocol parameters are summarized for experienced users in Table 19.

Table 19 Magnetic purification bead cleanup parameters after post-capture PCR

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

1. Prepare 400 µL of 70% ethanol per sample, plus excess, for use in step 8.
2. Mix the room-temperature purification beads well until homogeneous and consistent in color.
3. Add 50 µL of the purification bead suspension to each amplified library sample well, containing approximately 50 µL of streptavidin bead suspension.
4. Mix by pipetting up and down 15–20 times or cap the wells and vortex at high speed for 5–10 seconds then spin briefly to collect the samples, being careful not to pellet the beads.
5. Incubate the bead suspensions for 5 minutes at room temperature.
6. Put the strip tube into a magnetic separation device. Wait for the solution to clear (approximately 2 to 5 minutes).
7. Keep the 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 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 strip tube at room temperature for 2–5 minutes or until the residual ethanol has just evaporated.
13. Elute the library by adding 25 µL of Low TE buffer to each sample well.
14. Mix by pipetting up and down 10–15 times or cap the wells and vortex at high speed for 5 seconds. Verify that all beads have been resuspended, with no visible clumps in the suspension or bead pellets retained on the sides of the wells. If samples were vortexed, spin briefly to collect the liquid, being careful not to pellet the beads.
15. Incubate for 2–5 minutes at room temperature. Use of longer incubation times may improve recovery, especially for longer DNA or cDNA fragments.
16. Put the strip tube in the magnetic stand and leave until the solution is clear (up to 5 minutes).

17 Transfer the cleared supernatant (approximately 24 µL) to a fresh well. Keep on ice.

You can discard the beads at this time.

NOTE

If magnetic beads are carried over during the final elution step, samples can be placed on the magnetic stand while removing an aliquot for QC (below) and while removing an aliquot for pooling on page 23.

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

Step 7. QC and quantify final libraries

Analyze a sample of each library using one of the platforms listed in Table 20. Follow the instructions in the linked user guide provided for each assay.

Table 20 Post-capture library analysis options

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

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

Using the 150 bp to 1000 bp region of the electropherogram, determine the average fragment size and the library concentration. See Table 21 for fragment size distribution guidelines.

Table 21 Post-capture expected library fragment size guidelines

Hybridization input	Expected average fragment size (150–1000 bp region)
DNA library prepared from intact DNA fragmented for 2x100 NGS	350 to 450 bp
DNA library prepared from intact DNA fragmented for 2x150 NGS	380 to 480 bp
DNA library prepared from FFPE DNA fragmented for 2x100 or 2x150 NGS	250 to 390 bp
cDNA library prepared from intact RNA	380 to 480 bp
cDNA library prepared from FFPE RNA	250 to 390 bp

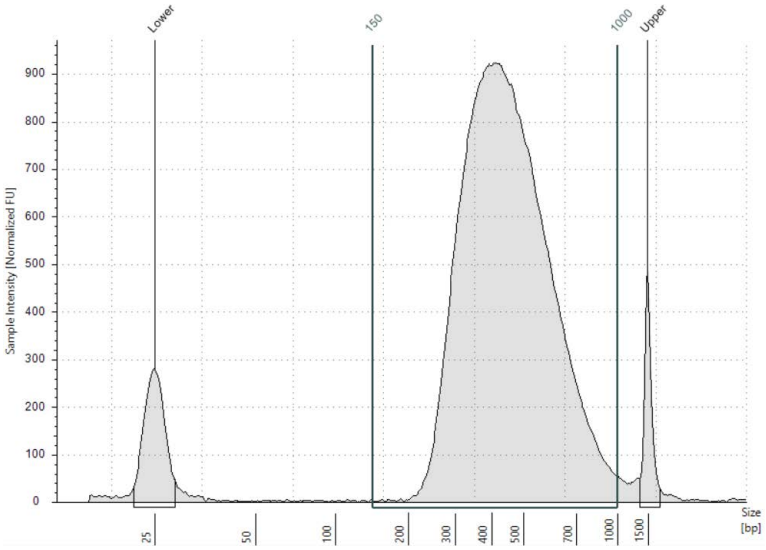

Figure 2 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

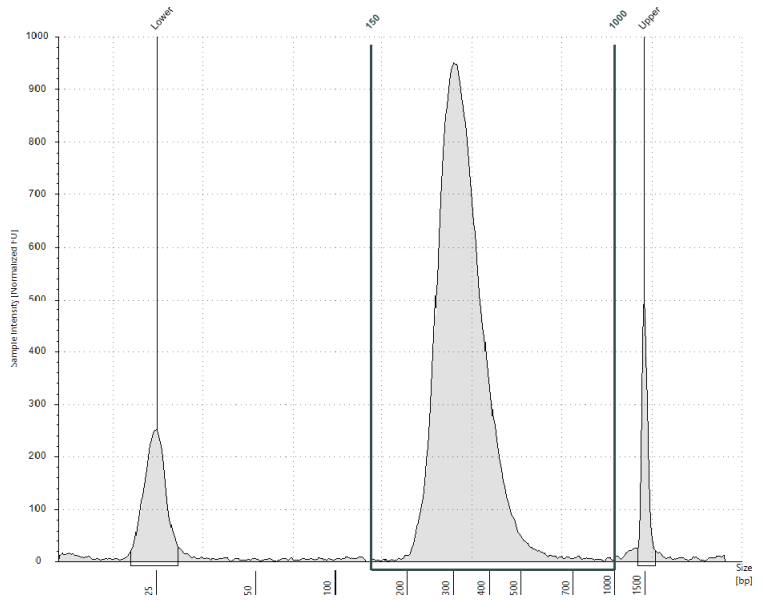

Figure 3 Post-capture library prepared from an FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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

Post-capture pooling guidelines

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the sequencer used, together with the amount of sequencing data required per sample for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample.

NOTE

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

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

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

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

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

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

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

is the number of indexes, and

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

Table 22 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM DNA or cDNA.

Table 22 Example of volume calculation for total volume of 20 µL at 10 nM concentration

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	20 µL	20 nM	10 nM	4	2.5
Sample 2	20 µL	10 nM	10 nM	4	5
Sample 3	20 µL	17 nM	10 nM	4	2.9
Sample 4	20 µL	25 nM	10 nM	4	2
Low TE					7.6

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

3
Appendix: NGS and Analysis Guidelines

This appendix provides guidelines for completing NGS using the Illumina platform and for SureSelect Max library read processing steps.

SureSelect Max Library Composition

The final SureSelect Max library fragments prepared using Agilent's Illumina-compatible kit modules are shown in Figure 4. Each fragment in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using Illumina sequencers. The libraries are ready for sequencing using standard Illumina paired-end primers and chemistry.

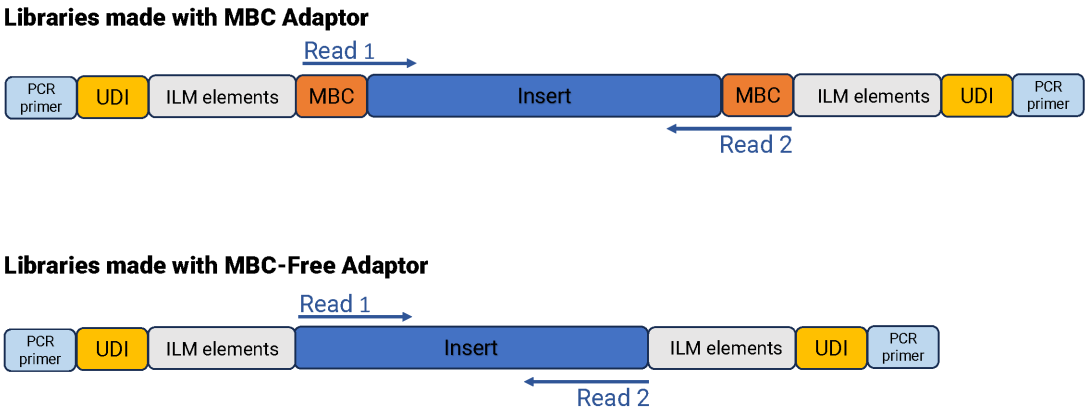

Figure 4 Content of SureSelect Max for ILM sequencing libraries. Each fragment contains one target insert (dark blue) surrounded by the following elements: Illumina paired-end sequencing elements (gray), unique dual indexes (gold), library PCR primers (light blue) and optional molecular barcode (MBC) sequences. MBCs include a 3-bp degenerate barcode plus dark bases.

Sequencing Setup and Run Guidelines

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. Table 23 provides guidelines for use of several Illumina instrument and chemistry combinations suitable for processing the SureSelect Max target-enriched NGS libraries. For other Illumina NGS platforms, consult Illumina's documentation for kit configuration and seeding concentration guidelines.

Table 23 Illumina Kit Configuration Selection Guidelines

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

Seeding concentration and cluster density may also need to be optimized based on the fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in Table 23 or provided by Illumina. Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Set up the sequencing run to generate Read 1 and Read 2 FASTQ files for each sample using the instrument's software in standalone mode or using an Illumina run management tool such as Local Run Manager (LRM), Illumina Experiment Manager (IEM) or BaseSpace. Enter the appropriate **Cycles** or **Read Length** value for your library read length and using 8-bp dual index reads. See Table 24 showing example settings for 2x150 bp sequencing.

Table 24 Run settings for 2x150 bp sequencing

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

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

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

- Each of the sample-level indexes (i7 and i5) requires an 8-bp index read. For complete index sequence information, see page 28.
- 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 MBC-tagged libraries, turn off any adaptor trimming tools included in Illumina's run setup and read processing software applications. Adaptors are trimmed in later processing steps to ensure proper processing of the degenerate MBC sequences in the adaptors.
- For runs set up using Illumina's LRM, IEM, or BaseSpace applications, refer to Illumina's instructions and support resources for setting up runs with custom library prep kits and index kits in the selected software. For use in these applications, the SureSelect Max index sequences can be obtained by downloading the SureSelect Max Index Sequence Resource Excel spreadsheet from Agilent.com. The provided sequences should be converted to .tsv/.csv file format or copied to a Sample Sheet according to Illumina's specifications for each application. If you need assistance with SureSelect Max run setup in your selected application (e.g., generating index files or Sample Sheet templates), contact the SureSelect support team (see page 2) or your local representative.

Analysis Pipeline Guidelines

Guidelines are provided below for typical NGS read processing and analysis pipeline steps appropriate for SureSelect Max DNA and RNA libraries. Your NGS 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. For MBC-tagged libraries, turn off the MBC/UMI trimming options in Illumina's demultiplexing software to allow proper adaptor processing and use of the MBCs by downstream tools. For MBC-free libraries, trimming tools in Illumina's demultiplexing software can be turned on.
- If you have prepared MBC-tagged libraries, but your sequence analysis pipeline excludes MBCs, you can remove MBCs during the demultiplexing step by trimming or masking the first five bases from each read. See page 41 for guidelines.
- For MBC-tagged reads, the demultiplexed FASTQ data needs to be pre-processed to trim the sequencing adaptors and to extract and use MBC sequences for de-duplication. Agilent's AGeNT toolkit, described on page 28, can be used for these pre-processing steps.

NOTE

Read pre-processing steps can also be completed using suitable open-source software tools, such as fgbio. Performance of open-source tools should be verified for appropriate adaptor and MBC sequence processing on both strands. Some non-Agilent adaptor trimmers may fail to remove the MBC sequences from the opposite adaptor, which may affect alignment quality.

AGeNT software guidelines for SureSelect Max DNA or RNA workflows

Agilent's AGeNT is a Java-based toolkit for library read processing steps, designed for users with bioinformatics expertise to enable building internal analysis pipelines. To download this toolkit, visit the AGeNT page at www.agilent.com. Use of the AGeNT read processing tools is outlined briefly below. See the AGeNT Best Practices document for more information. Instructions provided for the SureSelect XT HS2 platform are also applicable to the SureSelect Max platform.

- Prior to variant discovery, demultiplexed SureSelect Max library FASTQ data are pre-processed to remove sequencing adaptors and extract the MBC sequences (if present) using the AGeNT Trimmer module.
- The trimmed reads should be aligned (and MBC tags added to the aligned BAM file where applicable) using a suitable tool such as BWA-MEM for DNA libraries or STAR for RNA libraries.
- For MBC-containing libraries only, the AGeNT CReaK (Consensus Read Kit) tool is used to generate consensus reads and mark or remove duplicates for the aligned reads based on the read MBCs. CReaK includes consensus generation options suitable for DNA libraries (duplex, hybrid, or single-strand mode) and RNA libraries (single-strand mode).

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

RNA strandedness guidelines

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

SureSelect Max UDI Information

The SureSelect Max unique dual indexes (UDIs) are added to the library fragments during the library preparation workflow segment. Each fragment contains a unique 8-bp P5 and P7 index (see Figure 4 on page 25) suitable for Illumina sequencing platforms.

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

Index sequences are provided on page 30 through page 37. Index sequences can also be obtained by downloading the SureSelect Max Index Sequence Resource Excel spreadsheet from Agilent.com.

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

In Table 26 through Table 33 and in the downloadable Excel spreadsheet, P7 indexes are shown in forward orientation, applicable to any of the supported Illumina platforms. P5 indexes are shown in two orientations (forward and reverse complement) for use with different platforms and sequencing run setup and management tools, e.g., Local Run Manager and Instrument Run Setup. A selection of Illumina sequencing platforms and their P5 sequencing orientations are shown in Table 25. Correct representation of the P5 index orientation in sample sheets or during sequencing run setup is crucial to successful demultiplexing. Refer to Illumina support documentation and resources to determine the correct P5 index orientation for your application.

Table 25 P5 index sequencing orientation by Illumina platform

P5 Index Orientation	Platform
Forward	MiSeq
Reverse Complement*	NovaSeq 6000 with v1.5 chemistry NextSeq 500/550/1000/2000 iSeq 100 MiniSeq

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

SureSelect Max index sequences

Index sequences can also be obtained by downloading the SureSelect Max Index Sequence Resource Excel spreadsheet from Agilent.com.

Table 26 SureSelect Max Index Primer Pairs 1–48, provided in orange 96-well plate or in strip tubes

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

Table 27 SureSelect Max Index Primer Pairs 49–96, provided in orange 96-well plate

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

Table 28 SureSelect Max Index Primer Pairs 97–144, provided in blue 96-well plate

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

Table 29 SureSelect Max Index Primer Pairs 145–192, provided in blue 96-well plate

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

Table 30 SureSelect Max Index Primer Pairs 193–240, provided in green 96-well plate

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

Table 31 SureSelect Max Index Primer Pairs 241–288, provided in green 96-well plate

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

Table 32 SureSelect Max Index Primer Pairs 289–336, provided in red 96-well plate

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

Table 33 SureSelect Max Index Primer Pairs 337–384, provided in red 96-well plate

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

4
Reference

This section contains reference information, including Reagent Kit contents, troubleshooting information and a quick-reference protocol for experienced users.

Reagent Kit Contents

SureSelect Max Target Enrichment with overnight hybridization workflow uses the kits listed in Table 34. Detailed contents of each of the multi-part component kits are shown in Table 35 through Table 37.

Table 34 Kits for SureSelect Max Target Enrichment with Overnight Hybridization

SureSelect Max Kits and Included Component Kits	Component Kit Part Numbers		Storage Condition
	16 Hybridizations	96 Hybridizations	
SureSelect Max Overnight Hyb Kit (G9690A/G9690B)			
SureSelect Max Target Enrichment Kit Overnight Hyb Module, Box 1	5282-0132	5282-0134	Room Temperature
SureSelect Max Target Enrichment Kit Overnight Hyb Module, Box 2	5282-0133	5282-0135	–20°C
SureSelect Streptavidin Beads	5191-5741	5191-5742	+4°C
SureSelect Max Blockers and Primers Module for ILM (G9699A/G9699B)	5282-0136	5282-0137	–20°C
SureSelect Max Purification Beads (G9962A/G9962B)	5282-0225	5282-0226	+4°C

Component Kit Details

Table 35 SureSelect Max Target Enrichment Kit Overnight Hyb Module, Box 1 content

Kit Component	16 Hyb Kit (p/n 5282-0132)	96 Hyb Kit (p/n 5282-0134)
SureSelect Hyb 1	tube with orange cap	bottle
SureSelect Hyb 2	tube with red cap	tube with red cap
SureSelect Hyb 4	tube with black cap	tube with black cap
SureSelect Binding Buffer	bottle	bottle
SureSelect Wash Buffer 1	bottle	bottle
SureSelect Wash Buffer 2	bottle	bottle

Table 36 SureSelect Max Target Enrichment Kit Overnight Hyb Module, Box 2 content

Kit Component	16 Hyb Kit (p/n 5282-0133)	96 Hyb Kit (p/n 5282-0135)
SureSelect Hyb 3	tube with yellow cap	tube with yellow cap
SureSelect RNase Block	tube with purple cap	tube with purple cap
Amplification Master Mix	tube with red cap	bottle

Table 37 SureSelect Max Blockers and Primers Module for ILM content

Kit Component	16 Hyb Kit (p/n 5282-0136)	96 Hyb Kit (p/n 5282-0137)
Blocker Mix, ILM	tube with blue cap	tube with blue cap
SureSelect Post-Capture Primer Mix	tube with clear cap	tube with clear cap

Troubleshooting Guide

If vacuum concentrator is not available for library volume reduction prior to hybridization

✓The standard hybridization protocol requires addition of prepared library samples or library pools in a 4 µL volume, using a vacuum concentrator to reduce the volume of the library pools where needed. If a vacuum concentrator is not available in your laboratory, use the protocol modifications below to generate concentrated library pools for hybridization. This modification may result in some loss of library complexity.

Post-capture pooling workflow:

- Method 1: During the SureSelect Max Library Preparation protocol using Enzymatic Fragmentation or Covaris shearing, in the final pre-capture library preparation clean-up step reduce the amount of nuclease-free water used for elution from 15 µL to 6 µL.
- Method 2: If prepared libraries were already eluted in 15 µL, libraries can be concentrated using an additional round of magnetic bead purification. Follow the purification instructions on page 20, modifying step 3 to use a bead volume of 1.8X the available sample volume, then elute using 6 µL of nuclease-free water in step 13.

Pre-capture pooling workflow:

- For hybridization strip setup on page 12, make pools containing 2.5X amount of each library given in Table 7 on page 12. For example, for gDNA libraries enriched with the SureSelect XT PreCap Human All Exon V8 probe, pool 1250 ng of each of 8 indexed libraries for total pool of 10 µg library DNA.
- Concentrate each pool using an additional round of magnetic bead purification. Follow the purification instructions on page 20, modifying step 3 to use a bead volume of 1.8X the total sample volume.
- Elute in 8 µL of nuclease-free water in step 13. Place 4 µL of each concentrated pool in a separate well for the hybridization reaction. The remaining concentrated pool volume is not used in the hybridization reaction.

If yield of captured libraries is low

✓The protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance. Be sure to adhere to all instructions when setting up the reactions.

✓PCR cycle number may require optimization. Repeat target enrichment for the sample, increasing the post-capture PCR cycle number by 1 to 2 cycles.

✓The probe used for hybridization may have been compromised. Verify the expiration date on the probe vial or Certificate of Analysis. Adhere to the recommended storage and handling conditions. Ensure that the probe hybridization mix is prepared at the time of use, as directed on page 15, and that solutions containing the probe are not held at room temperature for extended periods.

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

- Adhere to all of the bead and reagent handling steps in the protocol. In particular, make sure to equilibrate beads at room temperature for at least 30 minutes before use, and use freshly-prepared 70% ethanol (prepared on day of use) for each purification procedure.
- Ensure that the magnetic beads are not over-dried just prior to sample elution (see step 12 on page 20). 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 or cDNA fragments.

If captured library fragment size is different than expected in electropherograms

✓Libraries prepared from FFPE DNA or RNA samples may have a smaller fragment size distribution due to the presence of fragments in the sample input that are smaller than the optimal fragment size for target enrichment. Adhere to the FFPE DNA or RNA quality guidelines provided in the SureSelect Max Library Preparation Module user guides.

✓Library fragment size selection during post-capture 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 and verify that you are using the bead volume recommended for post-capture purification on page 20.

If low percent on target is observed in library sequencing results

✓Stringency of post-hybridization washes may have been lower than required. Complete the wash steps as directed, paying special attention to the details of SureSelect Wash Buffer 2 washes listed below:

- Ensure that SureSelect Wash Buffer 2 is pre-warmed to 70°C before use (see page 17). Select a thermal cycler with a block configured for efficient heating of 0.2 mL liquid volumes; ensure that the plasticware containing the wash buffer is fully seated in the thermal cycler block wells, with minimal liquid volume visible above the block during the pre-warming step.
- Bead suspensions are mixed thoroughly during washes by pipetting up and down **and** vortexing (see page 17).

✓Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a vortex and plate spinner or centrifuge in close proximity to thermal cycler to retain the elevated sample temperature during mixing and transfer steps (step 8 to step 10 on page 15).

If your libraries are MBC-tagged but your sequence analysis pipeline excludes MBCs

✓You can remove the first 5 bases from Read 1 and Read 2 by masking or trimming before proceeding to downstream analysis using one of the options below:

- If demultiplexing using bcl2fastq, MBCs may be masked by including the base mask **N5Y*,I8,I8,N5Y*.** If demultiplexing using BCL Convert, MBCs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y*;I8;I8;N5Y*.** For both methods, ***** is replaced with value equal to the remaining read length after masking or trimming. For example, use **N5Y146,I8,I8,N5Y146** for 2x150 NGS with 151 cycles (as shown in Table 24 on page 26). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.
- The first 5 bases may be trimmed from the demultiplexed FASTQ files using the AGeNT Trimmer module while trimming adaptor sequences or using a suitable processing tool of your choice, such as seqtk. Non-Agilent adaptor trimmer performance should be verified for removal of the MBC sequences from the opposite adaptor (refer to Figure 4 on page 25); failure to remove MBC sequences from both strands may affect alignment quality.

Quick Reference Protocol: Max Overnight Hyb Target Enrichment

An abbreviated protocol summary is provided below for experienced users. Use the complete protocol on page 10 to page 21 until you are familiar with all of the protocol details.

Step	Summary of Conditions
	Hybridization (Day 1)
Place libraries in hyb wells and adjust volumes to 4 µL/well	Place prepared libraries or library pools into strip tube wells and adjust volume to 4 µL as below. **For post-cap pooling:** Use 12 µL of undiluted prepared library (recommended) or use at least 500 ng gDNA library or 200 ng cDNA library per well. **For pre-cap pooling:** Pool libraries into wells according to Table 7 on page 12. Reduce well volumes to <4 µL by vacuum concentration at ≤45°C. Bring well volumes to 4 µL with nuclease-free H_2O> vortex> spin> keep on ice.
Prep Overnight Hyb Buffer	**Per 8 Hyb reactions:** 66.3 µL Hyb 1 + 2.7 µL Hyb 2 + 26.5 µL Hyb 3 + 34.5 µL Hyb 4 **Per 24 reactions:** 185.6 µL Hyb 1 + 7.6 µL Hyb 2 + 74.2 µL Hyb 3 + 96.6 µL Hyb 4 Prepare at RT> mix> spin> keep at RT.
Program thermal cycler	Input the Blocking/Hybridization program in Table 38, using the temperature appropriate for your probe in segment 2. Pre-warm before loading samples.
Run pre-hybridization blocking protocol	Add 5 µL Blocker Mix ILM to each sample well; vortex> spin> place strip in cycler> resume program. Block at 67.5°C or 65°C for at least 5 min while preparing the remaining hyb reagents.
Prepare 25% RNAse Block dilution	**Per 8 Hyb reactions:** 4.5 µL RNAse Block + 13.5µL nuclease-free H_2O **Per 24 reactions:** 13 µL RNAse Block + 39 µL nuclease-free H_2O Prepare on ice> mix> spin> keep on ice.
Prepare Probe/Hyb Mix	Prepare the Probe/Hyb Mix for your probe design size–see Table 39. Prepare at RT> vortex> spin> keep briefly at RT.
Run the hybridization	Keep samples in cycler at 67.5°C/65°C while adding 20 µL Probe/Hyb Mix to wells> mix by pipetting. Seal wells completely> vortex> spin> return strip to cycler> hybridize 16 to 24 hours.

Table 38 Blocking/Hybridization program (29 µL vol; heated lid at 105°C)

Segment	Probe	Number of Cycles	Temperature (Purpose)	Time
1	All probes	1	95°C (Pre-blocking)	5 minutes
2	SureSelect XT Human All Exon V8/V8+UTR/V8+NCV, Human All Exon V7 or Clinical Research Exome V4	1	67.5°C (Blocking/Hyb)	Hold: Do blocking for ≥5 min Do hybridization for 16–24 hours
	All other SureSelect XT probe designs	1	65°C (Blocking/Hyb)	Hold: Do blocking for ≥5 min Do hybridization for 16–24 hours

Table 39 Preparation of Probe/Hyb Mix

	Probe designs ≥3 Mb			Probe designs<3 Mb		
Reagent	**Per Hyb Reaction**	**8 Hybs**	**24 Hybs**	**Per Hyb Reaction**	**8 Hybs**	**24 Hybs**
25% RNase Block solution	2 µL	18 µL	50 µL	2 µL	18 µL	50 µL
Probe	5 µL	45 µL	125 µL	2 µL	18 µL	50 µL
Prepared Overnight Hyb Buffer	13 µL	117 µL	325 µL	13 µL	117 µL	325 µL
Nuclease-free water	—	—	—	3 µL	27 µL	75 µL
Total	20 µL	180 µL	500 µL	20 µL	180 µL	500 µL

Step	Summary of Conditions
	Capture (Day 2)
Aliquot Wash Buffer 2	Aliquot 6 × 200 µL of SureSelect Wash Buffer 2 for each sample
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 and pre-warm beads/buffer at 68°C for 10 minutes.
Capture hybridized libraries	Add hybridized samples (~29 µL) to pre-warmed beads (200 µL) held in cycler> mix well by pipetting. Incubate capture strip in thermal cycler at 68°C for 10 minutes. During incubation, pre-warm the 6 × 200 µL aliquots of SureSelect Wash Buffer 2 to 70°C.
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 and vortexing 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.
	Post-capture amplification (Day 2)
Program thermal cycler	Input the appropriate thermal cycler program in Table 40. Pre-warm before loading samples.
Prepare Post-capture PCR Mix	**Per 8 Hyb reactions:** 225 µL Amplification Master Mix + 9 µL SureSelect Post-Capture Primer Mix **Per 24 reactions:** 650 µL Amplification Master Mix + 26 µL SureSelect Post-Capture Primer Mix Prepare on ice> mix> spin> keep on ice.
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.
Purify amplified libraries	50 µL amplified library bead suspension + 50 µL purification bead suspension> mix> incubate 5 min> collect beads> discard supernatant. Wash beads 2X with 70% ethanol. Elute in 25 µL Low TE Buffer> mix> incubate 2-5 minutes> collect beads> retain supernatant.
Quantify and qualify libraries	Analyze quantity and quality using TapeStation or Fragment Analyzer System

Table 40 Post-Capture PCR thermal cycler program (50 µL vol; heated lid at 105°C)

Segment	Number of Cycles			Temperature	Time
1	1			98°C	45 seconds
2	gDNA libraries (DNA input): Probes <0.2 Mb: 16 cycles Probes 0.2–3 Mb: 12–16 cycles Probes 3–5 Mb: 11–12 cycles Probes >5 Mb: 10–11 cycles	**OR**	cDNA libraries (RNA input): Probes <0.2 Mb: 16 cycles Probes 0.2–3 Mb: 14 cycles Probes 3–5 Mb: 13 cycles Probes >5 Mb: 12 cycles	98°C 60°C 72°C	15 seconds 30 seconds 30 seconds
3	1			72°C	1 minute
4	1			4°C	Hold

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In This Book

This guide provides instructions for SureSelect Max Target Enrichment using an Overnight Hybridization workflow including either post-capture or pre-capture pooling of NGS libraries.

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