SureSelect Max DNA Library Prep and Target Enrichment Automated using Agilent Bravo NGS Workstation (Option B)

With Enzymatic Fragmentation

For Illumina Platform NGS

Protocol

Version C0, January 2026

SureSelect platform manufactured with Agilent SurePrint technology.

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

Notices

Manual Part Number

G9660-90010

Edition

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Acknowledgment

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Or send an e-mail to:
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Agilent's world-wide Sales and Support Center contact details for your location can be obtained at www.agilent.com/en/contact-us/page.

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CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

In this Guide...

This guide provides an optimized protocol for preparation of target-enriched Illumina paired-end multiplexed sequencing libraries using SureSelect Max Enzymatic Fragmentation Library Preparation Kit and one of the SureSelect Max Hyb Kits (Fast or Overnight). Sample processing steps are automated using the NGS Workstation.

1 Before You Begin

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

2 Using the NGS Workstation for SureSelect Max Library Prep and Target Enrichment

This chapter contains an orientation to the Agilent Bravo NGS Workstation, an overview of the SureSelect Max DNA library prep (with enzymatic fragmentation) and target enrichment workflow and VWorks form, and considerations for designing SureSelect experiments for automated processing.

3 Preparation and Analysis of gDNA Samples

This chapter contains instructions for preparing genomic DNA samples to be used as input for the SureSelect Max library prep and target enrichment workflow.

4 Preparation of Purification Bead Plates

This chapter provides instructions on preparing all of the plates of SureSelect Max Purification Beads that are needed throughout the entire workflow. Each plate of SureSelect Max Purification Beads is prepared using a separate automation protocol available in the SureSelect Max DNA Utility VWorks form.

5 Enzymatic Fragmentation and Library Preparation

This chapter describes the steps for automated enzymatic fragmentation and library preparation of gDNA for the Illumina paired-read sequencing platform. Libraries can be prepared using adaptors that either include or do not include molecular barcodes (MBCs). For each sample to be sequenced, an individual dual-indexed library is prepared.

6 Hybridization Preparation

This chapter describes the steps to prepare the plate of DNA libraries or DNA library pools that will be used to set up the hybridization reactions.

7 Hybridization (Fast)

This chapter describes the steps to hybridize the DNA libraries or library pools to the Probe using the fast hybridization method.

8 Hybridization (Overnight)

This chapter describes the steps to hybridize the DNA libraries or library pools to the Probe using the overnight hybridization method.

9 Capture and Amplification

This chapter describes the steps to capture and wash hybridized gDNA, amplify and purify the captured libraries, assess quality and quantity of the captured libraries, and pool libraries for multiplexed sequencing.

10 NGS and Analysis Guidelines

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

11 Appendix

This appendix provides instructions for using the Aliquot_Libraries automation protocol to prepare a sample plate for single-plex hybridization.

12 Reference

This chapter contains reference information, including component kit contents, index sequences, and troubleshooting information.

What’s New in Version C0

- Fully updated **“Analysis Pipeline Guidelines"** on page 148.
- New *Note* below **Table 1** on page 13 explaining use of AMPure XP Beads as a replacement for SureSelect Max Purification Beads.
- Addition of the SureSelect Cancer CGP DNA Assay probe to list of recommended pre-designed probes for fast hybridization (**Table 2** on page 14).

What’s New in Version B0

- Updated volumes for resuspension of prepared SureSelect Streptavidin Beads. See **Table 83** on page 121.
- Added details to footnote in **Table 1** on page 13 regarding the quantity of SureSelect Max Purification Beads.

Content

1 Before You Begin

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

Make sure you have the most current protocol. Go to **www.agilent.com** and search for G9660-90010.

NOTE

This Protocol describes automated DNA sample processing for SureSelect Max DNA Library Prep (with enzymatic fragmentation) and Target Enrichment using the Agilent Bravo NGS Workstation (Option B).

For non-automated sample processing procedures, see the following Agilent publications:

G9660-90000 – SureSelect Max DNA Library Preparation with Enzymatic Fragmentation for ILM
G9689-90000 – SureSelect Max Target Enrichment with Fast Hybridization for ILM
G9690-90000 – SureSelect Max Target Enrichment with Overnight Hybridization for ILM

NOTE

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

Procedural Notes

- Certain protocol steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the NGS Workstation to allow rapid and efficient plate transfer.
- If your thermal cycler is compatible with the use of compression pads, add a compression pad whenever you load a plate that was sealed with the PlateLoc thermal microplate sealer. The pad improves contact between the plate and the heated lid of the thermal cycler.
- Prepare and load the NGS Workstation as detailed in each of the protocol steps before initiating each automated protocol run. When loading plates in the Labware MiniHub of the NGS Workstation, always place plates in the orientation shown in **Figure 6** on page 59.
- 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 best-practices to prevent PCR product contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR work areas and use dedicated supplies and reagents in each area. In particular, never use materials designated to post-PCR work for pre-PCR segments of the workflow. Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
  2. Maintain clean work areas. Clean pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution.
  3. 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.
- In general, follow Biosafety Level 1 (BSL1) safety rules.
- Possible stopping points, where samples may be stored at –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.

Safety Notes

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

SureSelect Max Modules and Probes Used in 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 protocol provides instructions for using the NGS Workstation to automate workflows for genomic DNA (gDNA) samples using the SureSelect Max Enzymatic Fragmentation Library Prep Kit and one of the compatible Hyb Kits (SureSelect Max Fast Hyb Kit or SureSelect Max Overnight Hyb Kit).

Table 1 contains the complete list of SureSelect Max reagent kits for use with this protocol. See **"Reagent Kit Contents"** on page 164 for a list of the contents of each SureSelect Max kit.

Table 2 is a list of recommended SureSelect probes. Make sure to select a probe that is compatible with your chosen hybridization workflow (fast or overnight).

Table 1 SureSelect Max reagent kits

Module Description	Agilent Part Number
Library Prep	
SureSelect Max Enzymatic Fragmentation Library Prep Kit, 96 Reactions	G9660B
SureSelect Max Adaptors and UDI Primers Kit for ILM (Select One):	
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 1-96	G9673A
MBC-Free Adaptors and UDI Primers 97-192	G9673B
MBC-Free Adaptors and UDI Primers 193-288	G9673C
MBC-Free Adaptors and UDI Primers 289-384	G9673D
SureSelect Max Purification Beads, 96 Reactions	G9962B (30 mL)*
Target Enrichment	
SureSelect Max Hyb Kit (Select One):	
SureSelect Max Fast Hyb Kit, 96 Reactions (for fast hybridization workflow)	G9689B
SureSelect Max Overnight Hyb Kit, 96 Reactions (for overnight hybridization workflow)	G9690B
SureSelect Max Blockers and Primers Kit for ILM	G9699B
SureSelect Max Purification Beads, 96 Reactions	G9962B (30 mL)*

* The SureSelect Max Purification Beads are needed for both the library prep and target enrichment steps. One bottle (30 mL) is sufficient for both steps when running up to 96 samples that are not pooled pre-capture. For 96 samples with pre-capture pooling, two bottles are required.

NOTE

AMPure XP Beads can replace SureSelect Max Purification Beads in the protocols provided in this user guide (see **Table 3** on page 15 for ordering information). Both types of magnetic purification beads have similar overall performance. The bead types are not, however, identical in composition and the average fragment length and yield results may differ slightly. Use a single bead source in validated protocols.

Table 2 Recommended Probes

Probe Description	Design ID	Ordering Information
Pre-designed Probes for Fast Hybridization Workflow		
SureSelect XT HS Human All Exon V8	S33266340	Select XT HS design probes for use in the SureSelect Max Fast Hybridization workflow. Visit the Agilent.com probe webpages or the SureDesign website to obtain ordering information. Select the appropriate formulation and format for your indexed library pooling workflow choice (post-capture or pre-capture pooling). Please contact Sales or your local representative if you need assistance.
SureSelect XT HS Human All Exon V8+UTR	S33613271	
SureSelect XT HS Human All Exon V8+NCV	S33699751	
SSel XT HS and XT Low Input Human All Exon V7	S31285117	
SureSelect XT HS Clinical Research Exome V4	S34226467	
SureSelect Cancer CGP DNA Assay	A3416642	
Pre-designed Probes for Overnight Hybridization Workflow		
SureSelect XT Human All Exon V8	S33266436	Select XT design probes for use in the SureSelect Max Overnight Hybridization workflow. Visit the Agilent.com probe webpages or the SureDesign website to obtain ordering information. Select the appropriate formulation and format for your indexed library pooling workflow choice (post-capture 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		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. Make sure to select the appropriate Hybridization option (**90 Minutes** or **Overnight**) for your workflow. 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.

NOTE

The 6-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 16 samples/pool (for 6 hybs × 16 samples/hyb). In order to have enough reagent to run 3 full columns (i.e., 24 wells), you must purchase 4 units of the 6-Hyb Probe.

The 12-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 8 samples/pool (for 12 hybs × 8 samples/hyb). In order to have enough reagent to run 3 full columns (i.e., 24 wells), you must purchase 2 units of the 12-Hyb Probe.

The 30-Hyb Probes support target enrichment for 480 samples pooled using the configuration recommended for the included designs of 16 samples/pool (for 30 hybs × 16 samples/hyb).

Additional Materials Required

Input genomic DNA samples

The SureSelect Max DNA library prep and target enrichment workflow is compatible with both high-quality gDNA prepared from fresh or fresh frozen samples and lower-quality DNA prepared from FFPE samples. Modifications required for FFPE samples are included throughout the protocol steps.

The workflow requires 10 ng to 200 ng of input DNA, with adjustments to DNA input amount or quantification method required for some FFPE samples. For optimal results, use the maximum amount of input DNA available within the recommended range. Analysis using the molecular barcodes is recommended when sample is available in low amounts (10–50 ng) or when detecting very low allele frequency variants using small probe designs.

See **Chapter 3**, "Preparation and Analysis of gDNA Samples" for instructions on preparing gDNA samples for the SureSelect Max workflow.

Additional reagents, materials, and equipment required

To determine the additional materials required for your unique needs, refer to the tables provided in the following sections.

Required Reagents and Equipment

Table 3 list the additional reagents that are required to complete this protocol.

Table 3 Additional Required Reagents

Description	Vendor and part number
AMPure XP Kit*	Beckman Coulter Genomics
60 mL	p/n A63881
450 mL	p/n A63882
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
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276
Qubit BR dsDNA Assay Kit	Thermo Fisher Scientific
100 assays	p/n Q32850
500 assays	p/n Q32853
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

* Optional alternative to SureSelect Max Purification Beads.

Required Equipment

Table 4 list the additional equipment that is required to complete this protocol.

Table 4 Additional Required Equipment

Description	Vendor and Part Number
Thermal cycler and accessories (including compression pads, if compatible)	Various suppliers *Important*: Not all PCR plate types are supported for use in the VWorks automation protocols for the NGS Workstation. Select a thermal cycler that is compatible with one of the supported PCR plate types. See supported plate types in the listing below.
PCR plates compatible with the NGS Workstation and associated VWorks automation protocols	Only the following PCR plates are supported: • 96 ABI PCR half-skirted plates (MicroAmp Optical plates), Thermo Fisher Scientific p/n N8010560 • 96 Agilent semi-skirted PCR plate, Agilent p/n 401334 • 96 Eppendorf twin.tec half-skirted PCR plates, Eppendorf p/n 951020303 • 96 Eppendorf twin.tec PCR plates (full-skirted), Eppendorf p/n 951020401
Qubit Fluorometer	Thermo Fisher Scientific p/n Q33238
Qubit Assay Tubes	Thermo Fisher Scientific p/n Q32856
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Microcentrifuge	Eppendorf microcentrifuge, model 5417C or equivalent
Plate centrifuge	Labnet International MPS1000 Mini Plate Spinner, p/n C1000 or equivalent
Magnetic separator	DynaMag-50 magnet, Thermo Fisher Scientific p/n 123-02D or equivalent
Conical tubes, sterile (50-mL)	Fisher Scientific p/n 352098 or equivalent
Conical tubes, sterile (15- and 50-mL)	Thermo Fisher Scientific p/n 352059 or equivalent
Multichannel pipette	Rainin Pipet-Lite Multi Pipette or equivalent
Single channel pipettes (10-, 20-, 200-, and 1000-µL capacity)	Rainin Pipet-Lite Pipettes or equivalent
Sterile, nuclease-free aerosol barrier pipette tips	general laboratory supplier
Vortex mixer	general laboratory supplier
Ice bucket	general laboratory supplier
Powder-free gloves	general laboratory supplier
Decontamination wipes	Dilute bleach (10%) wipes, e.g., Hype-Wipe Bleach Towelettes OR Isopropanol (70%) wipes, e.g., VWR Pre-Moistened Clean Wipes

Bravo NGS Workstation and Required Plasticware

Table 5 lists the ordering information for the NGS Workstation and the required plasticware for use with the NGS Workstation.

Table 5 Bravo NGS Workstation and Workstation plasticware

Description	Vendor and Part Number
Agilent Bravo NGS Workstation (Option B) Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5522A (VWorks software version 13.1.12.1543) OR Agilent p/n G5574AA (VWorks software version 13.1.12.1543)
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Eppendorf twin.tec full-skirted 96-well PCR plates	Eppendorf p/n 951020401 or 951020619
Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 19 mm height – used when NGS Workstation setup calls for **Agilent Shallow Well Reservoir**	Agilent p/n 201254-100
Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 44 mm height – used when NGS Workstation setup calls for **Agilent Deep Well Reservoir**	Agilent p/n 201244-100
Agilent Storage/Reaction Microplates, 96 wells, 1 mL/round well – used when NGS Workstation setup calls for **Agilent Deep Well Plate** or **Agilent DW Plate**	Agilent p/n 203426-100
Agilent Storage/Reaction Microplates, 96 wells, 2 mL/square well – used when NGS Workstation setup calls for **Waste Plate (Agilent 2 mL Square Well)**	Agilent p/n 201240-100

Recommended Nucleic Acid Analysis Options

Table 6 lists the options for nucleic acid analysis platforms.

Table 6 Nucleic Acid Analysis Platform Options -- Select One

Analysis System	Vendor and Part Number Information	Usage Notes
Agilent 4200/4150 TapeStation Instrument Consumables: 96-well sample plates 96-well plate foil seals 8-well tube strips 8-well tube strip caps D1000 ScreenTape D1000 Reagents	Agilent p/n G2991AA/G2992AA p/n 5042-8502 p/n 5067-5154 p/n 401428 p/n 401425 p/n 5067-5582 p/n 5067-5583	Recommended systems for QC of libraries prior to Target Enrichment (optional) and after Capture and Wash. Libraries may also be analyzed using the Agilent 2100 Bioanalyzer instrument with the DNA 1000 Kit, p/n 5067-1504, and High Sensitivity DNA Kit, p/n 5067-4626.
Agilent 5200/5300/5400 Fragment Analyzer Consumables: NGS Fragment Kit (1-6000 bp)	Agilent p/n M5310AA/M5311AA/M5312AA p/n DNF-473-0500	

Additional Materials Based on Sample Type

Table 7 lists additional required materials you may need depending on the DNA sample type.

Table 7 Additional Required Materials based on DNA Sample Type

Description	Vendor and Part Number
Required for preparation of high-quality DNA samples (not required for FFPE DNA sample preparation)	
High-quality gDNA purification system, for example: QIAamp DNA Mini Kit 50 Samples 250 Samples	 Qiagen p/n 51304 p/n 51306
Required for preparation of FFPE DNA samples (not required for high-quality DNA sample preparation)	
QIAamp DNA FFPE Tissue Kit, 50 Samples	Qiagen p/n 56404
Deparaffinization Solution	Qiagen p/n 19093
FFPE DNA integrity assessment system: Agilent NGS FFPE QC Kit 16 reactions 96 reactions **OR** TapeStation Genomic DNA Analysis Consumables: Genomic DNA ScreenTape Genomic DNA Reagents	 Agilent p/n G9700A p/n G9700B Agilent p/n 5067-5365 p/n 5067-5366

2 Using the NGS Workstation for SureSelect Max Library Prep and Target Enrichment

This chapter contains an orientation to the Agilent Bravo NGS Workstation, an overview of the SureSelect Max DNA library prep (with enzymatic fragmentation) and target enrichment workflow and VWorks form, and considerations for designing SureSelect experiments for automated processing.

About the Agilent Bravo NGS Workstation (Option B)

CAUTION Before you begin, make sure that you have read and understand operating, maintenance and safety instructions for using the Bravo platform and additional devices included with the NGS Workstation. Refer to the user guides listed in **Table 8**.

Review the user guides listed in **Table 8** (available at www.agilent.com) to become familiar with the general features and operation of the NGS Workstation components. Instructions for using the Bravo platform and other NGS Workstation components specifically for the SureSelect Max workflow are detailed in this user guide.

Table 8 NGS Workstation components User Guide reference information

Device	User Guide part number
Bravo Platform	SD-V1000376 (formerly G5562-90000)
VWorks Software	G5415-90068
BenchCel Microplate Handler	G5580-90000
Labware MiniHub	G5584-90001
PlateLoc Thermal Microplate Sealer	G5585-90010

About the Bravo Platform

The Bravo platform is a versatile liquid handler with a nine plate-location platform deck, suitable for handling 96-well, 384-well, and 1536-well plates. The Bravo platform is controlled by the VWorks Automation Control software. Fitted with a choice of three interchangeable disposable-tip pipette heads, it accurately dispenses fluids from 0.3 µL to 250 µL.

Bravo Platform Deck

The protocols in the following sections include instructions for placing plates and reagent reservoirs on specific Bravo deck locations. Use **Figure 1** to familiarize yourself with the location numbering convention on the Bravo platform deck.

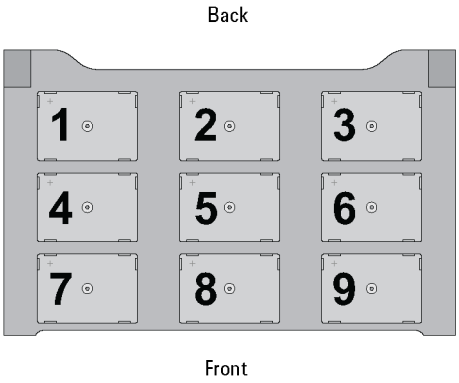


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

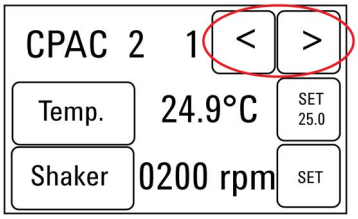
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the run.

Bravo deck heat block temperatures may be changed using the Inheco Multi TEC Control device touchscreen as described in the steps below. See **Table 9** for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

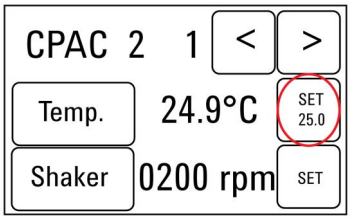
Table 9 Inheco Multi TEC Control touchscreen designations

Bravo Deck Position	Designation on Inheco Multi TEC Control Screen
4	CPAC 2 1
6	CPAC 2 2

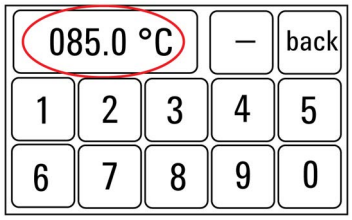
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



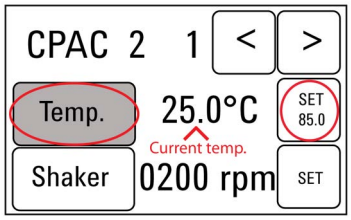
2 To set the temperature of the selected block, press the SET button.



3 Using the numeral pad, enter the desired temperature. The entered temperature appears in the top, left rectangle. Once the correct temperature is displayed, press the rectangle to enter the temperature.



4 Press the Temp button until the new temperature is displayed on the SET button and until the Temp button is darkened, indicating that the selected heat block is heating or cooling to the new temperature setting. The current temperature of the block is indicated in the center of the display.



Setting the Temperature of Bravo Deck Position 9 Using the ThermoCube Device

Bravo deck position 9 is equipped with a ThermoCube thermoelectric temperature control system, used to incubate components at a defined temperature during the run. During protocols that require temperature control at position 9, you will be instructed to start and set the temperature of the ThermoCube device before starting the run.

ThermoCube temperature settings are modified using the control panel (LCD display screen and four input buttons) on the front panel of the device using the following steps.

1 Turn on the ThermoCube and wait for the LCD screen to display TEMP.

2 Press the UP or DOWN button to change SET TEMP 1 to the required set point.

3 Press the START button.

The ThermoCube then initiates temperature control of Bravo deck position 9 at the displayed set point.

VWorks Automation Control Software

VWorks software, included with your NGS Workstation, allows you to control the robot and integrated devices using a PC. The NGS Workstation is preloaded with VWorks software containing all of the necessary SureSelect system liquid handling protocols. General instructions for starting up the VWorks software and the included protocols are provided below. Each time a specific VWorks protocol is used in the SureSelect procedure, any settings required for that protocol are included in the relevant section of this manual.

NOTE

The instructions in this manual are compatible with VWorks software version 13.1.12.1543.

If you have questions about VWorks version compatibility, please contact service.automation@agilent.com.

Logging in to the VWorks software

1. Double-click the VWorks icon or the SureSelect Max DNA VWorks Form shortcut on the Windows desktop to start the VWorks software.
2. If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
3. In the User Authentication dialog, type your VWorks user name and password, and click **OK**. (If no user account is set up, contact the administrator.)

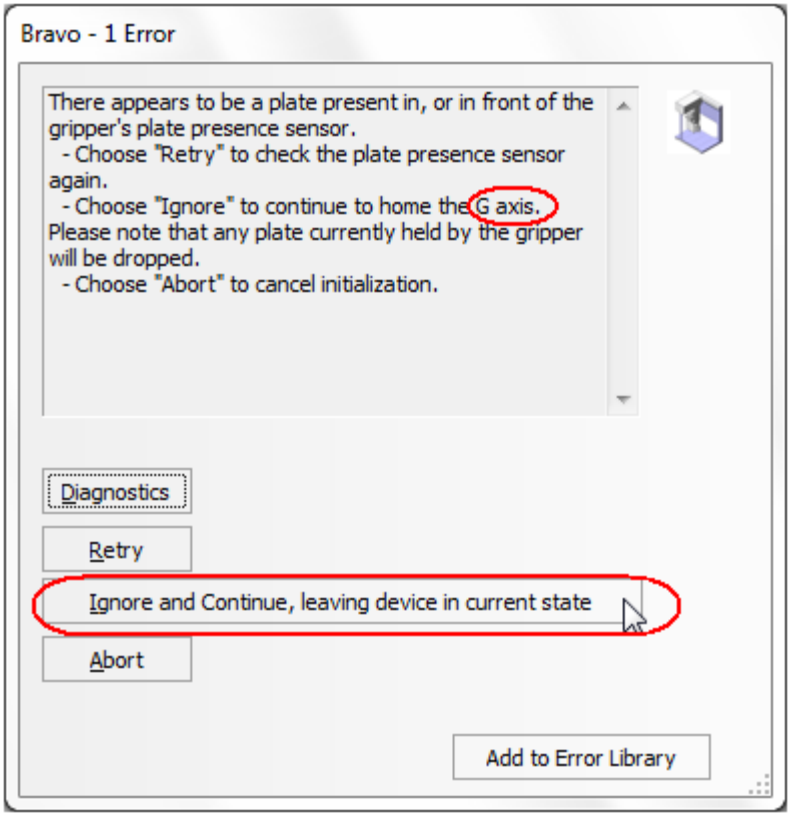
VWorks protocol and runset files

VWorks software uses two file types for automation runs, *.pro* (protocol) files and *.rst* (runset) files. Runset files are used for automated procedures in which the NGS Workstation uses more than one automation protocol during the run.

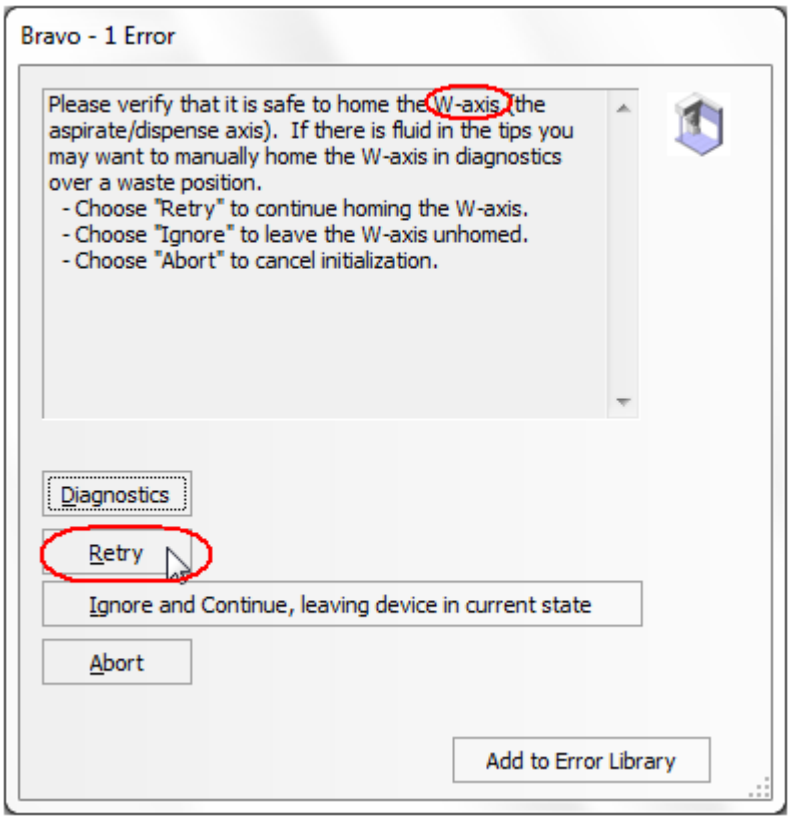
Error messages encountered at start of run

After starting the run, you may see the error messages displayed below. When encountered, make the indicated selections and proceed with the run. Encountering either or both of these error messages is not indicative of a problem with the NGS workstation or your run setup.

1 If you encounter the G-axis error message shown below, select **Ignore and Continue**, leaving device in current state.



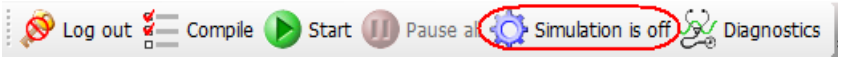
2 If you encounter the W-axis error message shown below, select **Retry**.



Verifying the Simulation setting

VWorks software may be run in simulation mode, during which commands entered on screen are not completed by the NGS Workstation. If workstation devices do not respond when you start a run, verify the simulation mode status in VWorks using the following steps.

1 Verify that **Simulation is off** is displayed on the status indicator (accessible by clicking V**iew > Control Toolbar**).

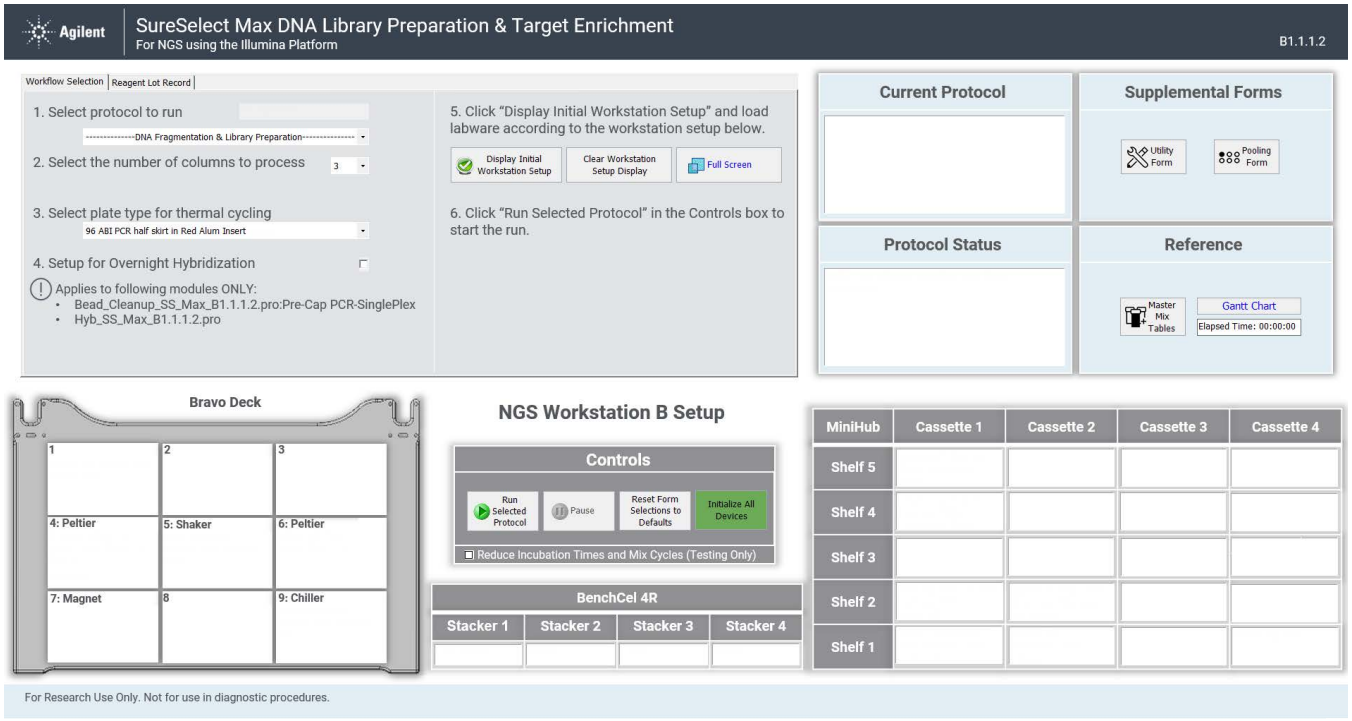


2 If the indicator displays **Simulation is on**, click the status indicator button to turn off the simulation mode.

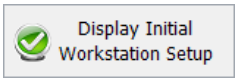
NOTE If you cannot see the toolbar above the SureSelect Max DNA VWorks form, click the **Full Screen** button to exit full screen mode. If the toolbar is still not visible, right-click on the form and then select **Control Toolbar** from the menu.

Using the SureSelect Max DNA Form to Set Up and Start a Run

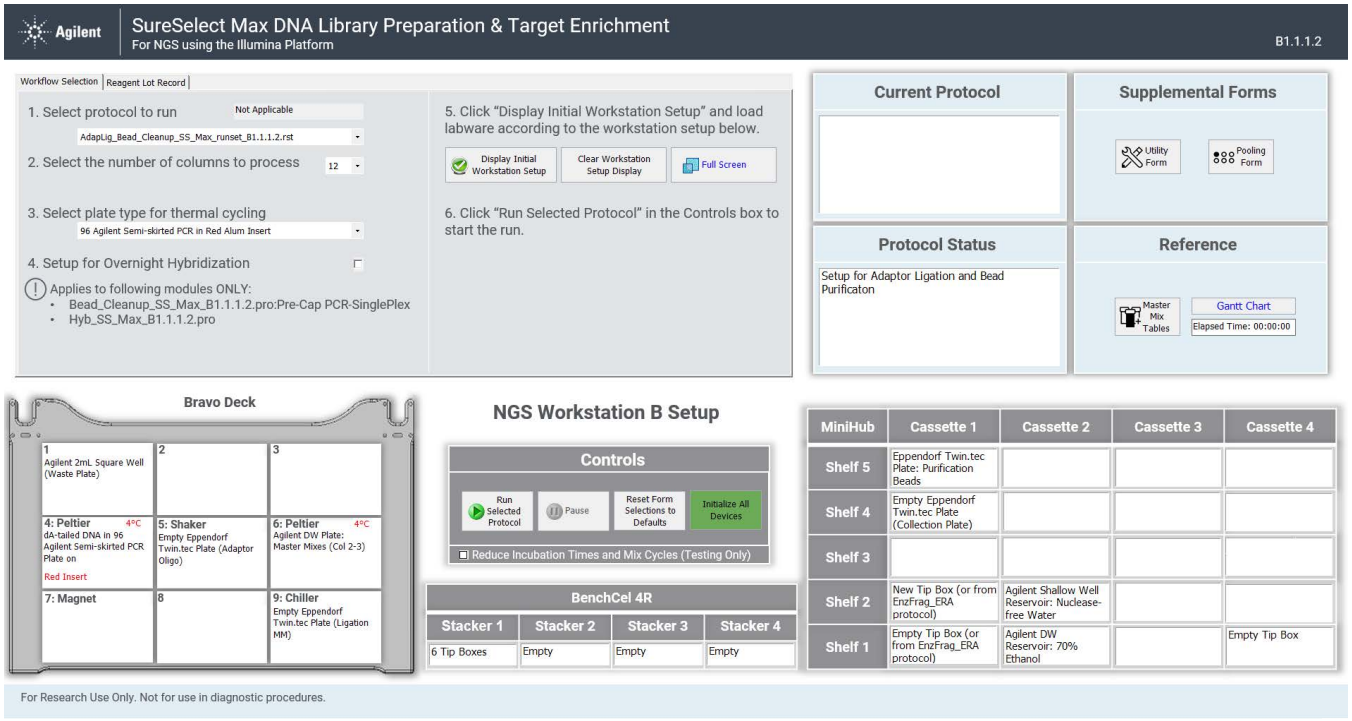
Use the SureSelect Max DNA VWorks form, shown below, to set up and start each SureSelect automation protocol or runset.



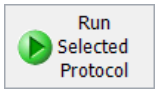
1. Open the form using the shortcut on your desktop, or by opening the file **Agilent_SS_Max_DNA_B1.1.1.2.VWForm** in the directory **C:\VWorks Workspace\NGS Option B\Agilent_SS_Max_DNA_B1.1.1.2\Forms**.
2. Use the form drop-down menus to select the appropriate SureSelect workflow step and number of columns of samples for the run.
3. Once all run parameters have been specified on the form, click **Display Initial Workstation Setup**.



The NGS Workstation B Setup region of the form will then display the required placement of reaction components and labware in the NGS Workstation for the specified run parameters.

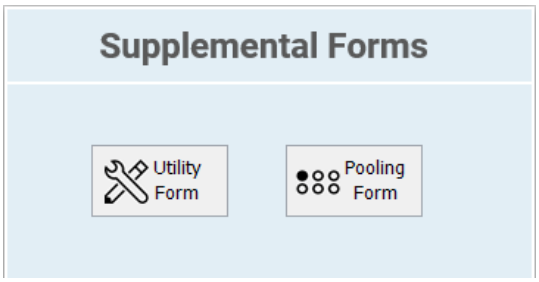


4 After verifying that the NGS Workstation has been set up correctly, click **Run Selected Protocol**.



Accessing the Supplemental VWorks Forms

The SureSelect Max DNA VWorks form provides buttons, shown below, for accessing two supplemental forms: the Utility form and the Pooling and Normalization form.



Overview of the Utility form

To open the Utility form from the SureSelect Max DNA VWorks form, click **Utility Form** in the Supplemental Forms section. Once you are viewing the Utility form, you can return to the SureSelect Max DNA VWorks form by clicking **SSEL Max DNA Form** in the Supplemental Forms section.

The Utility form (**Figure 2**) provides automated protocols to perform a variety of basic liquid-handling tasks used throughout the workflow. The tasks executed by these protocols can be, if desired, performed manually (i.e., without the use of an automated protocol) without significantly increasing the hands-on time of the workflow. Descriptions of the available protocols are provided in **Table 10** on page 35.

- The top half of the form has a variety of tools and controls (drop-down lists, buttons, etc.) for selecting the automation protocol, selecting the number of columns in the run, selecting the plate type, starting or pausing a run, and accessing resources.
- The bottom half of the form has graphic representations of the Bravo deck (positions 1 through 9), the BenchCel microplate handler (stackers 1 through 4), and the Labware MiniHub (Cassettes 1 through 4 and shelves 5 through 1).

After using the top of the form to make the necessary selections for the run, click **Display Initial Workstation Setup** to populate the graphics on the bottom of the form with the required setup for the Bravo deck, BenchCel, and LabWare MiniHub.

Detailed setup instructions for the protocols on the Utility form are included as part of the instructions in the subsequent chapters in this user guide.

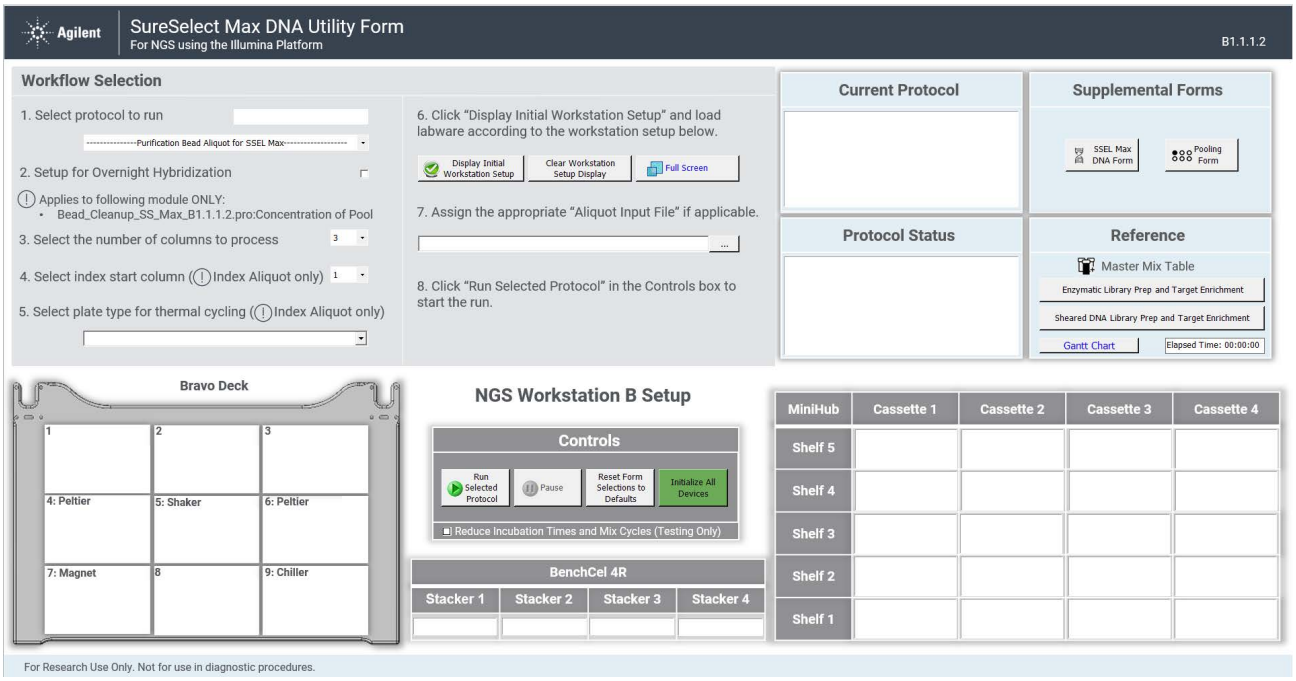


Figure 2 Utility form for SureSelect Max DNA

Overview of the Pooling and Normalization form

To open the Pooling and Normalization form from the SureSelect Max DNA VWorks form, click **Pooling Form** in the Supplemental Forms section. Once you are viewing the Pooling and Normalization form, you can return to the SureSelect Max VWorks form by clicking **SSEL Max DNA Form** in the Supplemental Forms section.

The Pooling and Normalization form (**Figure 3**) provides an automated protocol for pooling 8 or 16 samples for the pre-capture pooling workflow. The pooling protocol is the only protocol offered through the Pooling and Normalization form.

- The top of the form has a variety of tools and controls (drop-down lists, buttons, etc.) for setting the pooling parameters, selecting the plate type, starting or pausing a run, and accessing resources.
- The bottom of the form has graphic representations of the Bravo deck (positions 1 through 9), the BenchCel microplate handler (stackers 1 through 4), and the Labware MiniHub (Cassettes 1 through 4 and shelves 5 through 1).

After using the top of the form to make the necessary selections for the run, click **Display Initial Workstation Setup** to populate the graphics at the bottom of the form with the required setup for the Bravo deck, BenchCel, and Labware MiniHub.

Detailed setup instructions for the Pooling and Normalization form are included as part of the instructions in the **"Hybridization Preparation"** chapter.

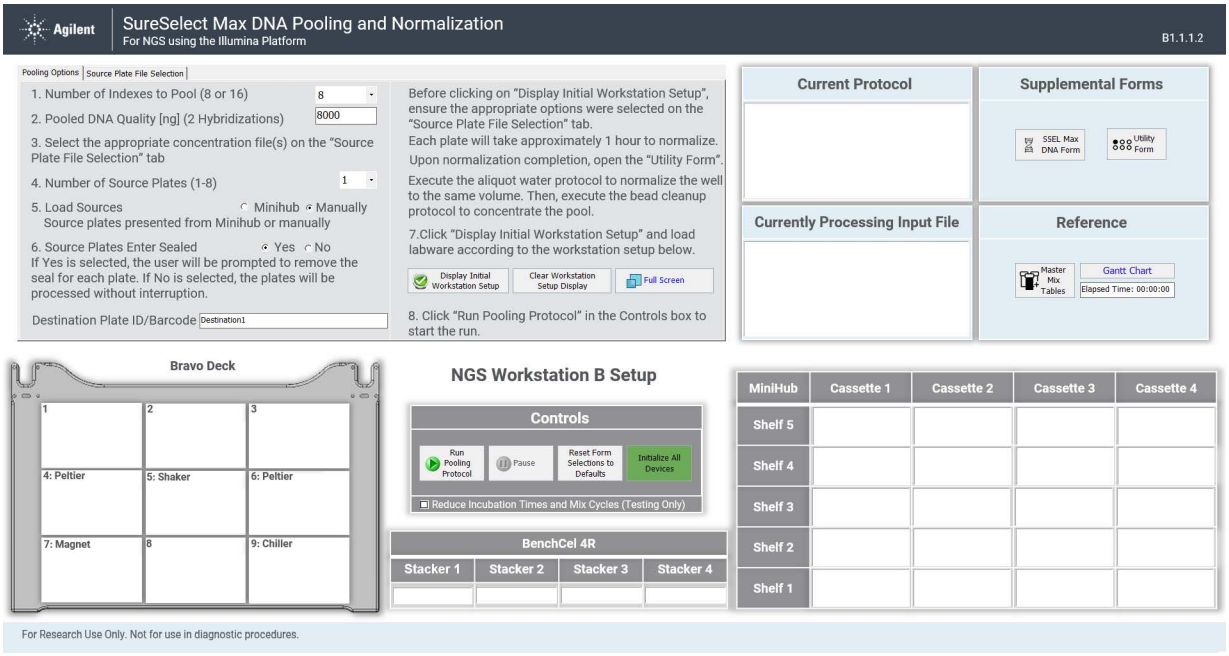


Figure 3 Pooling and Normalization form for SureSelect Max DNA

Overview of the SureSelect Max DNA Library Prep (with Enzymatic Fragmentation) and Target Enrichment Procedure

Figure 4 summarizes the SureSelect Max DNA library preparation and target enrichment workflow for DNA samples to be sequenced using the Illumina paired-read sequencing platform. For each sample, the workflow includes enzymatic fragmentation, library preparation, hybridization, and captures.

Agilent offers eight different SureSelect Max Adaptors and UDI Primers Kit for ILM to allow for multiplexed sequencing (refer to **"Index Primer Pair Plate Maps"** on page 166). You can pool samples for multiplexed sequencing either prior to hybridization with the Probe (i.e., pre-capture pooling) or after hybridization and subsequent PCR amplification of the captured libraries (i.e., post-capture pooling).

See **Table 10** for a summary of the VWorks protocols used during the workflow. Then, see **Preparation and Analysis of gDNA Samples**, **Preparation of Purification Bead Plates**, **Enzymatic Fragmentation and Library Preparation**, **Hybridization Preparation**, **Hybridization (Fast)**, **Hybridization (Overnight)**, and **Capture and Amplification** chapters for complete instructions for use of the VWorks protocols for sample processing.

The SureSelect Max DNA library preparation protocol is compatible with both high-quality gDNA prepared from fresh or fresh frozen samples and lower-quality DNA prepared from FFPE samples, using a DNA input range of 10 to 200 ng DNA.

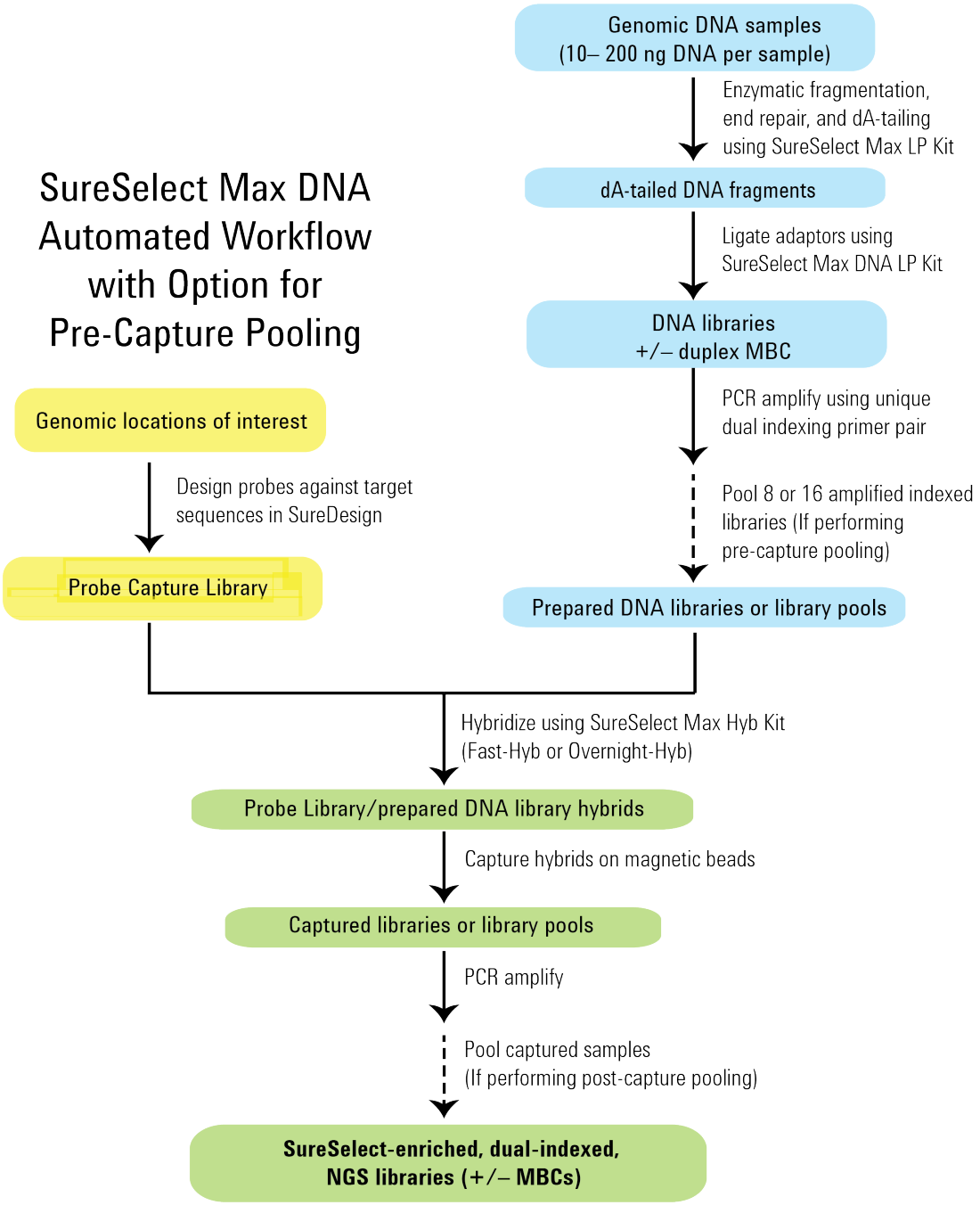


Figure 4 Overall sequencing sample preparation workflow

Automation Protocols used in the Workflow

Table 10 Overview of VWorks protocols and runsets

Workflow Step	Substep	VWorks Protocols/Runsets Used for NGS Workstation automation
Purification Beads Aliquoting (SureSelect Max Purification Beads or AMPure XP Beads)	Aliquot beads for use in the Adaptor Ligation runset	PurificationBead_Aliquot (Library Prep w/ Enz Frag)*
	Aliquot beads for use in the Pre-Capture PCR purification protocol	PurificationBead_Aliquot (Pre-Capture PCR)*
	Aliquot Beads for use in the Pre-Capture Pooling protocol for concentrating the DNA	PurificationBead_Aliquot (Concentration of Pool)*
	Aliquot Beads for use in the Post-Capture PCR purification protocol	PurificationBead_Aliquot (Post-Capture PCR)*
Fragmentation and dA-Tailing	Shear DNA samples using enzymatic fragmentation, repair DNA ends, and add dA-tailing	EnzFrag_ERA_SS_Max
Library Preparation	Ligate adaptors (with or without MBCs) to DNA fragments; purify duplex DNA libraries with beads	AdapLig_Bead_Cleanup_SS_Max_runset
	Aliquot the SureSelect Max UDI Primers into the pre-capture PCR plate	Index_Aliquot*
	Amplify indexed DNA libraries with unique dual indexing primer pair	Pre-CapPCR_SS_Max
	Purify indexed DNA libraries using beads in preparation for multi-plex hybridization	Bead_Cleanup_SS_Max (Pre-Cap PCR - MultiPlex)
	Purify indexed DNA libraries using beads in preparation for single-plex hybridization	Bead_Cleanup_SS_Max (Pre-Cap PCR - SinglePlex)
	Analyze indexed DNA libraries using Agilent TapeStation platform	TS_D1000*
Library Pooling (for **pre-capture pooling** workflow)	Pool indexed DNA libraries in pools of 8 or 16	PreCapture_Pooling†
Multi-Plex Pre-Hybridization (for **pre-capture pooling** workflow)	Dilute pooled samples of indexed DNA libraries to normalize volumes to 100 µL	Aliquot_Water*
	Concentrate pooled samples to either 8 µL (overnight hyb) or 24 µL (fast hyb) for hybridization	Bead_Cleanup_SS_Max (Concentration of Pool)*
Optional: Single-Plex Pre-Hybridization (for **post-capture pooling** workflow with **Fast hyb**)	Aliquot desired volume of library	Aliquot_Libraries*
Hybridization and Capture	Hybridize prepped libraries or library pools (target enrichment)	Hyb_SS_Max
	Capture and wash DNA hybrids	Capture&Wash_SS_Max

Table 10 Overview of VWorks protocols and runsets (continued)

Workflow Step	Substep	VWorks Protocols/Runsets Used for NGS Workstation automation
Post-Capture Sample Processing	Amplify target-enriched libraries or library pools	Post-CapPCR_SS_Max
	Purify amplified libraries or library pools using beads	Bead_Cleanup_SS_Max (Post-Capture PCR)
	Analyze final libraries or library pools using Agilent TapeStation platform	TS_HighSensitivity_D1000*
	For post-capture pooling workflow, pool indexed DNA libraries	Aliquot_Captures*

* This protocol is set up and executed from the SureSelect Max DNA Utility Form in VWorks.

† This protocol is set up and executed from the SureSelect Max DNA Pooling and Normalization Form in VWorks.

Workflow Modulations

The SureSelect Max DNA library preparation (with enzymatic fragmentation) and target enrichment workflow can be modulated for different applications as described below and summarized in **Table 11** on page 37.

DNA Sample Integrity Protocols are compatible with both high-quality gDNA prepared from fresh or fresh frozen samples and lower-quality DNA prepared from FFPE samples with minor protocol modifications.

MBC Usage The automated SureSelect Max DNA workflow supports library preparation with either MBC-tagged adaptors or MBC-free adaptors. Agilent offers SureSelect Max Adaptors and UDI Primers Kit for ILM for both types of adaptors in the adaptor oligo mix.

Sample Pooling Options The automated SureSelect Max DNA workflow supports two different approaches for sample pooling.

- Pre-capture pooling – Following PCR amplification of the indexed DNA libraries, pool either 8 or 16 of the libraries (depending on Probe design) prior to hybridization with the Probe. Each library pool is then hybridized with the Probe.
- Post-capture pooling – Following hybridization with the Probe and subsequent PCR amplification of the captured libraries, pool multiple indexed libraries together prior to sequencing. The allowable number of libraries per pool is dependent on the output specifications of the sequencing platform and the amount of sequencing data required.

Hybridization Time The automated SureSelect Max DNA workflow can prepare hybridization reactions for either an overnight hybridization or a fast hybridization. These methods use different SureSelect probes and different SureSelect Max Hyb Kits. They also require a different setup on the VWorks form.

- Overnight hybridization – The hybridization step of the thermal cycler program is 16 to 24 hours.
- Fast hybridization – The hybridization step of the thermal cycler program is 1 to 2 hours.

Table 11 Summary of workflow modulations supported by the automation protocols

Property	Options	Usage Notes
DNA Sample Integrity	Intact DNA	Use standard protocol with 10-200 ng input DNA
	FFPE DNA	Qualify DNA before use in assay; see **"Preparation and qualification of gDNA from FFPE samples"** on page 42 for guidance.
MBC Usage	MBC-tagged Adaptors	For library preparation, use a SureSelect Max Library Prep Kit that contains MCB-tagged adaptors in the adaptor oligo mix. Refer to **Table 1** on page 13.
	MBC-free Adaptors	For library preparation, use a SureSelect Max Library Prep Kit that contains MCB-free adaptors in the adaptor oligo mix. Refer to **Table 1** on page 13.
Pooling Strategy	Pre-Capture Pooling	The SureSelect Max DNA Library Prep and Hyb Kits are suitable for both pre-capture and post-capture pooling workflows. Where needed, this user guide provides differentiated instructions for pre-capture and post-capture pooling. Follow the instructions that pertain to your chosen pooling strategy.
	Post-Capture Pooling	

Table 11 Summary of workflow modulations supported by the automation protocols (continued)

Property	Options	Usage Notes
Hybridization Time	Fast	For target enrichment, use the SureSelect Max Fast Hyb Kit (see **Table 1** on page 13) and a Probe designed for fast hybridization (see **Table 2** on page 14). In the VWorks form, make sure that the check box next to **Setup for Overnight Hybridization** is NOT marked. 
	Overnight	For target enrichment, use a SureSelect Max Overnight Hyb Kit and a Probe designed for overnight hybridization (see **Table 2** on page 14). In the VWorks form, make sure that the check box next to **Setup for Overnight Hybridization** is marked. 

Experimental Setup Considerations for Automated Runs

Agilent SureSelect Automated Library Prep and Capture System runs may include 1, 2, 3, 4, 6, or 12 columns (equivalent to 8, 16, 24, 32, 48, or 96 wells) of gDNA samples to be enriched for sequencing on the Illumina platform. Plan your experiments using complete columns of samples.

Table 12 Columns to Samples Equivalency

Number of Columns Processed	Total Number of Samples Processed
1	8
2	16
3	24
4	32
6	48
12	96

The number of columns or samples that may be processed using the supplied reagents (see **Table 3**) will depend on the experimental design. For greatest efficiency of reagent use, plan experiments using at least 3 columns per run. Each 96-reaction kit contains sufficient reagents for 96 reactions configured as 4 runs of 3 columns of samples per run.

Considerations for Placement of gDNA Samples in 96-well Plates for Automated Processing

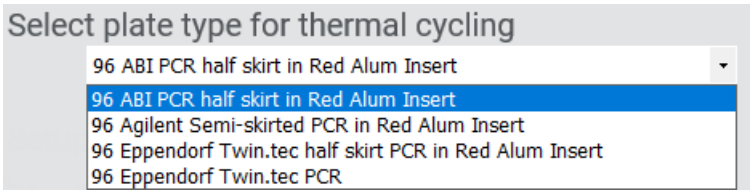
- The NGS Workstation processes samples column-wise beginning at column 1. The gDNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- At the hybridization step (see **Figure 4**), you can add a different Probe to each row of the plate. Plan your experiment such that each prepared DNA library corresponds to the appropriate probe row in the sample plate.
- For post-capture amplification (see **Figure 4**), different probes can require different amplification cycle numbers, based on the probe design sizes. It is most efficient to process similar-sized probes on the same plate. See **Table 89** on page 127 to determine which probes may be amplified on the same plate.

Considerations for Equipment Setup

- Some workflow steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the NGS Workstation to allow rapid and efficient plate transfer.
- Several workflow steps require that the sample plate be sealed using the PlateLoc thermal microplate sealer included with the NGS Workstation, and then centrifuged to collect any dispersed liquid. To maximize efficiency, locate the centrifuge in close proximity to the NGS Workstation.

PCR Plate Type Considerations

Automation protocols include several liquid-handling steps in which reagents are dispensed to PCR plates in preparation for transfer to a thermal cycler. For these steps, you must specify on the VWorks Form which PCR plate type will be used to allow correct configuration of the liquid handling components. Before you begin the automation protocol, make sure that you are using a supported PCR plate type. The PCR plate type to be used in the protocol is specified using the menu below. Vendor and part number information is provided for the supported plate types in **Table 13**.



CAUTION

The plates listed in **Table 13** are compatible with the NGS Workstation and associated VWorks automation protocols, designed to support use of various thermal cyclers.

Do not use PCR plates that are not listed in **Table 13**, even if they are compatible with your chosen thermal cycler.

Table 13 Ordering information for supported PCR plates

Description in VWorks menu	Vendor and part number
96 ABI PCR half-skirted plates (MicroAmp Optical plates)	Thermo Fisher Scientific p/n N8010560
96 Agilent semi-skirted PCR plate	Agilent p/n 401334
96 Eppendorf twin.tec half-skirted PCR plates	Eppendorf p/n 951020303
96 Eppendorf twin.tec PCR plates (full-skirted)	Eppendorf p/n 951020401

3 Preparation and Analysis of gDNA Samples

This chapter contains instructions for preparing genomic DNA samples to be used as input for the SureSelect Max library prep and target enrichment workflow.

The workflow requires 10 ng to 200 ng of input sample DNA. See **"Input genomic DNA samples"** on page 15 for more information on requirements and recommendations for the input samples.

Preparation of high-quality gDNA from fresh biological samples

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

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

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

Additional qualification of DNA samples is not required for DNA derived from fresh biological samples. Store the gDNA samples on ice for same-day library preparation, or at –20°C for later processing.

Preparation and qualification of gDNA from FFPE samples

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

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

2 Assess the quality (DNA integrity) for each FFPE DNA sample using one of the methods below. Store the gDNA samples on ice for same-day library preparation, or at –20°C for later processing.

Option 1: Qualification using the Agilent NGS FFPE QC Kit (Recommended Method)

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include a ΔΔCq DNA integrity score and the precise quantity of amplifiable DNA in the sample, allowing direct normalization of DNA input for each sample. DNA input recommendations based on ΔΔCq scores for individual samples are summarized in **Table 14**.

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

b Remove a 1 µL aliquot of the FFPE gDNA sample for analysis using the Agilent NGS FFPE QC Kit to determine the ΔΔCq DNA integrity score. See the kit user manual (G9700-90000) at www.agilent.com for more information.

c For all samples with ΔΔCq DNA integrity score ≤1, use the Qubit-based gDNA concentration determined in **step a**, above, to determine volume of input DNA needed for the protocol.

d For all samples with ΔΔCq DNA integrity score >1, use the qPCR-based concentration of amplifiable gDNA, reported by the Agilent NGS FFPE QC Kit results, to determine amounts of input DNA for the protocol.

Table 14 DNA input guidelines based on ΔΔCq DNA integrity score

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

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

Option 2: Qualification using Agilent's Genomic DNA ScreenTape assay DIN score

Agilent's Genomic DNA ScreenTape assay, used in conjunction with Agilent's 4200 TapeStation, provides a quantitative electrophoretic assay for DNA sample integrity determination. This assay reports a DNA Integrity Number (DIN) score for each sample which is used to estimate the appropriate normalization of DNA input required for low-integrity DNA samples.

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

b Remove a 1 µL aliquot of the FFPE gDNA sample and analyze using the Genomic DNA ScreenTape assay. See the user manual at www.agilent.com for more information.

c Using the DIN score reported for each sample in the Genomic DNA ScreenTape assay, consult **Table 15** to determine the recommended amount of input DNA for the sample.

Table 15 SureSelect Max DNA input modifications based on DNA Integrity Number (DIN) score

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

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

NOTE

DNA quality affects the recommended pre-capture PCR cycle number, as outlined in **Table 36** on page 69. Samples with differing PCR cycle number requirements must be processed in separate library preparation runs.

4 Preparation of Purification Bead Plates

This chapter provides instructions on preparing all of the plates of SureSelect Max Purification Beads that are needed throughout the entire workflow. Each plate of SureSelect Max Purification Beads is prepared using a separate automation protocol available in the SureSelect Max DNA Utility VWorks form.

Preparing the plates of SureSelect Max Purification Beads at the start of the workflow allows you to execute the remainder of the workflow with fewer delays between steps. Importantly, however, if you are running the workflow over multiple days, only prepare the plates of SureSelect Max Purification Beads that are to be used within the day and the following day. *Do not prepare SureSelect Max Purification Bead plates more than one day in advance of when they are needed.* Also, make sure to label the plates (without writing on the plates themselves) to properly differentiate them.

NOTE The SureSelect Max Purification Beads are stored at +4°C. Equilibrate the beads at room temperature for at least 30 minutes before use, then vortex to mix. You can substitute the SureSelect Max Purification Beads with AMPure XP beads.

Step 1. Prepare the bead plate to be used for library prep

The AdapLig_Bead_Cleanup_SS_Max_runset requires a bead plate containing 60 µL of beads in each well. Use the PurificationBead_Aliquot (Library Prep w/ Enz Frag) protocol to prepare the bead plate needed for the adaptor ligation runset.

Prepare the NGS Workstation and reagents for the PurificationBead_Aliquot (Library Prep w/ Enz Frag) protocol

1 Clear the Labware MiniHub and BenchCel of all plates and tip boxes.

2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.

3 Prepare an Agilent shallow well reservoir containing the SureSelect Max Purification Beads suspension.

   a Verify that the SureSelect Max Purification Beads suspension is at room temperature.

   b Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.

   c Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the AdapLig_Bead_Cleanup_SS_Max runset (each column accommodates 8 gDNA samples).

Load the NGS Workstation

4 Load the Bravo deck according to **Table 16**.

Table 16 Initial Bravo deck configuration for PurificationBead_Aliquot (Library Prep w/ Enz Frag) protocol

Location	Content
2	New tip box
5	Empty Eppendorf twin.tec plate
6	Reservoir of SureSelect Max Purification Beads suspension prepared in **step 3**
8	Empty tip box

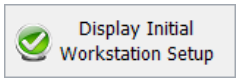
Run VWorks protocol PurificationBead_Aliquot (Library Prep w/ Enz Frag)

5 In the VWorks software, open the Utility form. See **"Accessing the Supplemental VWorks Forms"** on page 30.

6 Under **Select protocol to run**, select the **PurificationBead_Aliquot (Library Prep w/ Enz Frag)** protocol.

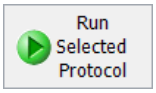
7 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

8 Click **Display Initial Workstation Setup**.



9 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

10 When verification is complete, click **Run Selected Protocol**.



Running the PurificationBead_Aliquot (Library Prep w/ Enz Frag) protocol takes approximately 5 minutes. The protocol directs the NGS Workstation to aliquot 60 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent Deep Well plate.

11 When the protocol is complete, remove the Agilent Deep Well plate containing the SureSelect Max Purification Beads suspension from position 5 of the Bravo deck.

12 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the AdapLig_Bead_Cleanup_SS_Max runset (refer to **Table 30** on page 64).

If desired, pour any unused bead suspension still present in the Agilent shallow well reservoir back into the original stock bottle.

Step 2. Prepare the bead plate to be used for Pre-Capture PCR Purification

The Bead_Cleanup_SS_Max (Pre-Cap PCR) protocols require a bead plate containing 50 µL of beads in each well. Use the PurificationBead_Aliquot (Pre-Capture PCR) protocol to prepare the bead plate needed for purification of pre-capture PCR products.

Prepare the NGS Workstation and reagents for the PurificationBead_Aliquot (Pre-Capture PCR) protocol

1 Clear the Labware MiniHub and BenchCel of all plates and tip boxes.

2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.

3 Prepare an Agilent shallow well reservoir containing the SureSelect Max Purification Beads suspension.

   a Verify that the SureSelect Max Purification Beads suspension is at room temperature.

   b Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.

   c Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol (each column accommodates 8 amplified DNA samples).

Load the NGS Workstation

4 Load the Bravo deck according to **Table 17**.

Table 17 Initial Bravo deck configuration for PurificationBead_Aliquot (Pre-Capture PCR) protocol

Location	Content
2	New tip box
5	Empty Eppendorf twin.tec plate
6	Reservoir of SureSelect Max Purification Beads suspension prepared in **step 3**
8	Empty tip box

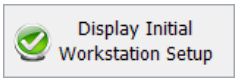
Run VWorks protocol PurificationBead_Aliquot (Pre-Capture PCR)

5 In the VWorks software, open the Utility form. See **"Accessing the Supplemental VWorks Forms"** on page 30.

6 Under **Select protocol to run**, select the **PurificationBead_Aliquot (Pre-Capture PCR)** protocol.

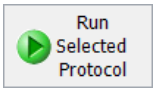
7 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

8 Click **Display Initial Workstation Setup**.



9 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

10 When verification is complete, click **Run Selected Protocol**.



Running the PurificationBead_Aliquot (Pre-Capture PCR) protocol takes approximately 5 minutes. The protocol directs the NGS Workstation to aliquot 50 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent Deep Well plate.

11 When the protocol is complete, remove the Agilent Deep Well plate containing the SureSelect Max Purification Beads suspension from position 5 of the Bravo deck.

12 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol (refer to **Table 41** on page 75).

If desired, pour any unused bead suspension still present in the Agilent shallow well reservoir back into the original stock bottle.

Step 3. Prepare the bead plate to be used for concentration of pooled DNA (pre-capture pooling workflow only)

The Bead_Cleanup_SS_Max (Concentration of Pool) protocol is part of the pre-capture pooling workflow. It requires a bead plate containing 180 µL of beads in each well. Use the PurificationBead_Aliquot (Concentration of Pool) protocol to prepare the bead plate needed for concentrating the DNA library pools.

The bead plate for the Bead_Cleanup_SS_Max (Concentration of Pool) protocol is only needed if you are running the pre-capture pooling workflow option. If you are using the post-capture pooling workflow, proceed to **"Step 4. Prepare the bead plate to be used for Post-Capture Purification"** on page 52.

Prepare the NGS Workstation and reagents for the PurificationBead_Aliquot (Concentration of Pool) protocol

1 Clear the Labware MiniHub and BenchCel of all plates and tip boxes.

2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.

3 Prepare an Agilent shallow well reservoir containing the SureSelect Max Purification Beads suspension.

   a Verify that the SureSelect Max Purification Beads suspension is at room temperature.

   b Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.

   c Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the Bead_Cleanup_SS_Max (Concentration of Pool) protocol (each column accommodates 8 DNA library pools).

Load the NGS Workstation

4 Load the Bravo deck according to **Table 18**.

Table 18 Initial Bravo deck configuration for PurificationBead_Aliquot (Concentration of Pool) protocol

Location	Content
2	New tip box
5	Empty Agilent Deep Well Plate
6	Reservoir of SureSelect Max Purification Beads suspension prepared in **step 3**
8	Empty tip box

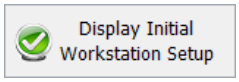
Run VWorks protocol PurificationBead_Aliquot (Concentration of Pool)

5 In the VWorks software, open the Utility form. See **"Accessing the Supplemental VWorks Forms"** on page 30.

6 Under **Select protocol to run**, select the **PurificationBead_Aliquot (Concentration of Pool)** protocol.

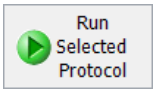
7 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

8 Click **Display Initial Workstation Setup**.



9 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

10 When verification is complete, click **Run Selected Protocol**.



Running the PurificationBead_Aliquot (Concentration of Pool) protocol takes approximately 5 minutes. The protocol directs the NGS Workstation to aliquot 180 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent Deep Well plate.

11 When the protocol is complete, remove the Agilent Deep Well plate containing the SureSelect Max Purification Beads suspension from position 5 of the Bravo deck.

12 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the Bead_Cleanup_SS_Max (Concentration of Pool) protocol (refer to **Table 55** on page 92).

If desired, pour any unused bead suspension still present in the Agilent shallow well reservoir back into the original stock bottle.

Step 4. Prepare the bead plate to be used for Post-Capture Purification

The Bead_Cleanup_SS_Max (Post-Capture PCR) protocol requires a bead plate containing 50 µL of beads in each well. Use the PurificationBead_Aliquot (Post-Capture PCR) protocol to prepare the bead plate needed for purification of post-capture PCR products.

Prepare the NGS Workstation and reagents for the PurificationBead_Aliquot (Post-Capture PCR) protocol

1 Clear the Labware MiniHub and BenchCel of all plates and tip boxes.

2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.

3 Prepare an Agilent shallow well reservoir containing the SureSelect Max Purification Beads suspension.

   a Verify that the SureSelect Max Purification Beads suspension is at room temperature.

   b Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.

   c Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the Bead_Cleanup_SS_Max (Post-Capture PCR) protocol (each column accommodates 8 indexed libraries).

Load the NGS Workstation

4 Load the Bravo deck according to **Table 19**.

Table 19 Initial Bravo deck configuration for PurificationBead_Aliquot (Post-Capture PCR) protocol

Location	Content
2	New tip box
5	Empty Eppendorf twin.tec plate
6	Reservoir of SureSelect Max Purification Beads suspension prepared in **step 3**
8	Empty tip box

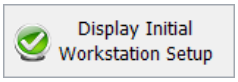
Run VWorks protocol PurificationBead_Aliquot (Post-Capture PCR)

5 In the VWorks software, open the Utility form. See **"Accessing the Supplemental VWorks Forms"** on page 30.

6 Under **Select protocol to run**, select the **PurificationBead_Aliquot (Post-Capture PCR)** protocol.

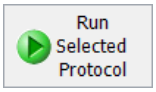
7 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

8 Click **Display Initial Workstation Setup**.



9 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

10 When verification is complete, click **Run Selected Protocol**.



Running the PurificationBead_Aliquot (Post-Capture PCR) protocol takes approximately 5 minutes. The protocol directs the NGS Workstation to aliquot 50 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent Deep Well plate.

11 When the protocol is complete, remove the Agilent Deep Well plate containing the SureSelect Max Purification Beads suspension from position 5 of the Bravo deck.

12 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the Bead_Cleanup_SS_Max (Post-Capture PCR) protocol (refer to **Table 96** on page 132).

If desired, pour any unused bead suspension still present in the Agilent shallow well reservoir back into the original stock bottle.

5 Enzymatic Fragmentation and Library Preparation

This chapter describes the steps for automated enzymatic fragmentation and library preparation of gDNA for the Illumina paired-read sequencing platform. Libraries can be prepared using adaptors that either include or do not include molecular barcodes (MBCs). For each sample to be sequenced, an individual dual-indexed library is prepared.

The SureSelect Max DNA library prep and target enrichment workflow requires 10 ng to 200 ng of input sample DNA. See **"Input genomic DNA samples"** on page 15 for more information on requirements and recommendations for the input samples. For an overview of the SureSelect Max DNA library prep and target enrichment workflow, see **Figure 4** on page 34.

Step 1. Enzymatically Fragment the DNA, Repair Ends, and dA-Tail

In this step, the NGS Workstation completes the liquid-handling steps for setting up the reactions for enzymatic fragmentation, end repair, and dA-tailing of the DNA samples using the EnzFrag_ERA_SS_Max automated protocol. After the NGS Workstation completes the liquid-handling steps, you transfer the PCR plate to a thermal cycler for incubation.

This step of the workflow uses the components listed in **Table 20**. Prepare each component as directed.

Table 20 Reagents thawed before use in EnzFrag_ERA_SS_Max

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect Max Enzymatic Fragmentation Library Preparation Module, stored at –20°C	Frag/A-Tail Buffer (yellow cap)	Thaw on ice then keep on ice, vortex to mix.	**page 58**
	Frag/A-Tail Enzyme Mix (green cap)	Place on ice just before use. Mix thoroughly by inversion at least 5X.	**page 58**

Prepare the NGS Workstation for EnzFrag_ERA_SS_Max protocol

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
5. Place a red PCR plate insert at Bravo deck position 4.

Pre-program the thermal cycler for the Frag/A-Tail reaction

1 Pre-program a thermal cycler using the program in **Table 21**. Set the heated lid to 105°C. Hold at 4°C until samples are added.

Table 21 Thermal cycler program for Frag/A-Tail (50 µL)

Step	Temperature	Time
Step 1	4°C	Hold
Step 2	37°C	Varies -- see **Table 22**
Step 3	65°C	30 minutes
Step 4	4°C	Hold

Optimal fragmentation conditions may vary based on the NGS read length to be used in the workflow. Refer to **Table 22** below for the duration at 37°C appropriate for the quality of the input DNA and the required NGS read length.

Table 22 Fragmentation duration based on DNA quality and NGS read length

Input type	NGS read length requirement	Duration of 37°C incubation step (step 2 of thermal cycler program above)
Intact DNA	2 × 100 reads	20 minutes
	2 × 150 reads	10 minutes
FFPE DNA	2 × 100 reads OR 2 × 150 reads	15 minutes

Prepare the source plates for EnzFrag_ERA_SS_Max protocol

Prepare the sample plate

1 In the wells of the PCR plate, dilute 10–200 ng of each gDNA sample with nuclease-free water or 1X Low TE Buffer to a final volume of 40 µL. Use the PCR plate that is to be placed in the thermal cycler for the fragmentation/dA-tailing program.

See**Table 14** or **Table 15** for FFPE DNA input guidelines based on the measured DNA quality in each sample.

NOTE

Do not substitute the 1X Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) with standard TE buffer containing >0.1 mM EDTA; higher EDTA concentrations inhibit the fragmentation reaction.

Prepare the Frag/A-Tail master mix

2 Prepare the appropriate volume of Frag/A-Tail master mix by combining the reagents in **Table 23**.

Seal the tube and vortex at high speed for 5–10 seconds. Spin briefly to remove any bubbles and keep on ice.

Table 23 Preparation of Frag/A-Tail master mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Frag/A-Tail Buffer (yellow cap)	4 µL	79.9 µL	117.3 µL	128.0 µL	170.7 µL	255.9 µL	491.3 µL
Frag/A-Tail Enzyme Mix (green cap)	6 µL	119.9 µL	176.0 µL	192.0 µL	256.0 µL	383.9 µL	737.0 µL
Total Volume	**10.0 µL**	**199.8 µL**	**293.3 µL**	**320.0 µL**	**426.7 µL**	**639.8 µL**	**1228.3 µL**

Prepare the Frag/A-Tail master mix source plate

3 Prepare the **Agilent Deep Well** master mix source plate for the run as indicated in **Table 24**. Add the indicated volume of the Frag/A-Tail master mix to all wells of column 1 of the plate. Keep the master mix on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 5**.

Table 24 Preparation of the master mix source plate for EnzFrag_ERA_SS_Max protocol

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Frag/A-Tail master mix	Column 1 (A1-H1)	23.5 µL	34.5 µL	39.5 µL	52.7 µL	78.5 µL	144.5 µL

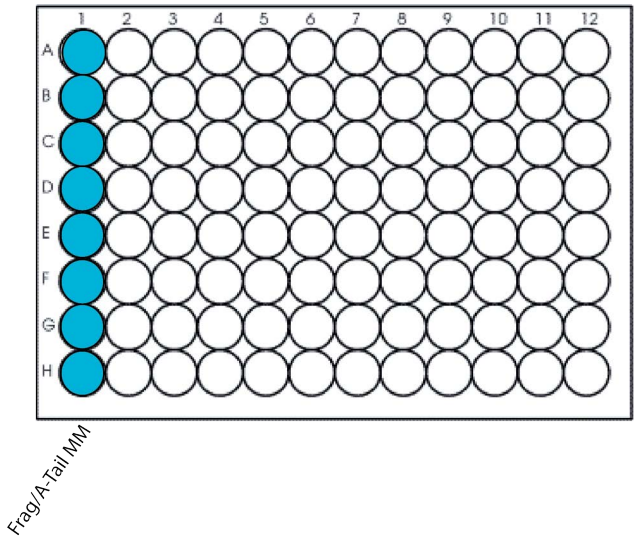


Figure 5 Configuration of the **Agilent Deep Well** master mix source plate for protocol EnzFrag_ERA_SS_Max

4 Seal the master mix source plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

5 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles. Keep the master mix source plate on ice.

NOTE

The presence of bubbles in source plate solutions may cause inaccurate volume transfer by the Bravo liquid handling platform. Ensure that the source plate is sealed and centrifuged prior to use in a run.

Load the NGS Workstation for EnzFrag_ERA_SS_Max protocol

1 Load the Labware MiniHub according to **Table 25**, using the plate orientations shown in **Figure 6**.

Table 25 Initial MiniHub configuration for EnzFrag_ERA_SS_Max protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	—	—	—	—
Shelf 1 (Bottom)	Empty tip box	—	—	Empty tip box

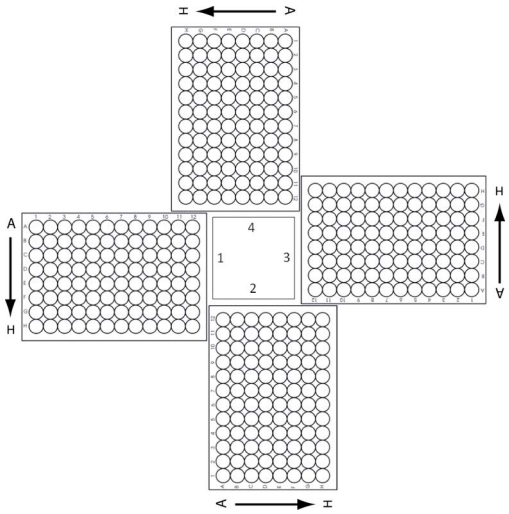


Figure 6 Agilent Labware MiniHub plate orientation.

2 Load the Bravo deck according to **Table 26**.

Table 26 Initial Bravo deck configuration for EnzFrag_ERA_SS_Max protocol

Location	Content
4	gDNA sample plate prepared on **page 57**; seated in red insert (PCR plate type must be specified on setup form under Parameter 3)
5	Empty Eppendorf twin.tec plate
6	Frag/A-Tail master mix source plate, unsealed

3 Load the BenchCel Microplate Handling Workstation according to **Table 27**.

Table 27 Initial BenchCel configuration for EnzFrag_ERA_SS_Max protocol

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	2 Tip boxes	—	—	—
2	2 Tip boxes	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	2 Tip boxes	—	—	—
12	2 Tip boxes	—	—	—

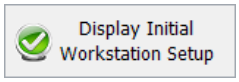
Run the VWorks EnzFrag_ERA_SS_Max protocol

1 On the SureSelect setup form, under **Select protocol to run**, select the **EnzFrag_ERA_SS_Max** protocol.

2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

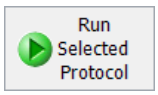
3 Under **Select plate type for thermal cycling**, select the specific type of PCR plate used at position 4 of the Bravo deck.

4 Click **Display Initial Workstation Setup**.



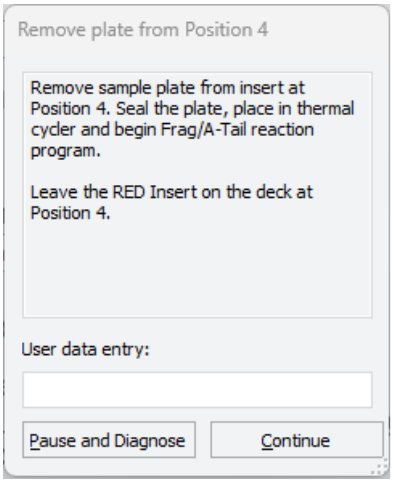
5 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

6 When verification is complete, click **Run Selected Protocol**.



Running the EnzFrag_ERA_SS_Max protocol takes approximately 15 minutes. Once complete, the samples are ready for fragmentation and dA-tailing (performed in the pre-programmed thermal cycler). The samples are located in the PCR plate at position 4 of the Bravo deck.

7 When you see the following prompt, remove the PCR plate from position 4 of the Bravo deck and seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing setting of 165°C and 1.0 seconds.



8 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

9 Immediately place the sample plate in the thermal block. Close the lid and resume the thermal cycler program in **Table 21** on page 57.

10 From the Bravo deck, remove the Agilent Deep Well plate that was used as the Frag/A-Tail master mix source plate from position 9 and set it aside. You will use this same plate again in the next automation protocol.

11 While the thermal cycler program is running, initiate the steps in the next section, **"Step 2. Ligate Adaptors to Fragmented DNA"**. When instructed to load the Bravo deck for the Adaptor Ligation runset, you will transfer the PCR plate of fragmented/dA-tailed DNA samples from the thermal cycler to the Bravo deck (see **Table 31** on page 65).

Step 2. Ligate Adaptors to Fragmented DNA

In this step, the NGS Workstation completes the liquid-handling steps for adaptor ligation (with or without MBCs) and bead purification using the AdapLig_Bead_Cleanup_SS_Max automated runset.

This step uses the aliquoted plate of SureSelect Max Purification Beads that was prepared on **page 46**.

This step also uses the components listed in **Table 28**. Prepare each component as directed.

Table 28 Reagents thawed before use in AdapLig_Bead_Cleanup_SS_Max_runset

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect Max Enzymatic Fragmentation Library Preparation Module, stored at –20°C	Ligation Master Mix (bottle)	Thaw on ice then keep on ice. Mix thoroughly by inversion at least 5X.	**page 63**
SureSelect Max Adaptors and UDI Primers Kit for ILM, stored at –20°C	For MBC-tagged libraries: SureSelect Max MBC Adaptor Oligo Mix, ILM (white cap) OR For MBC-free libraries: SureSelect Max MBC-Free Adaptor Oligo Mix (black cap)	Thaw on ice then keep on ice, vortex to mix.	**page 63**

Prepare the NGS Workstation for AdapLig_Bead_Cleanup_SS_Max runset

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
5. Place a red PCR plate insert at Bravo deck position 4.

Prepare the master mix source plate for AdapLig_Bead_Cleanup_SS_ Max runset

1 Prepare the **Agilent Deep Well** master mix source plate containing the Ligation Master Mix and Adaptor Oligo Mix. Add the volumes indicated in **Table 29** of each mixture to all wells of the indicated column of the Agilent Deep Well plate. Keep the reagents on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 7**.

Table 29 Preparation of the master mix source plate for AdapLig_Bead_Cleanup_SS_Max_runset

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Ligation Master Mix (bottle)	Column 2 (A2-H2)	34.5 µL	55.5 µL	71.6 µL	95.5 µL	139.5 µL	267.0 µL
SureSelect Max MBC Adaptor Oligo Mix, ILM (white cap) OR SureSelect Max MBC-Free Adaptor Oligo Mix (black cap)	Column 3 (A3-H3)	17.5 µL	22.9 µL	21.6 µL	28.8µL	42.7µL	79.2 µL

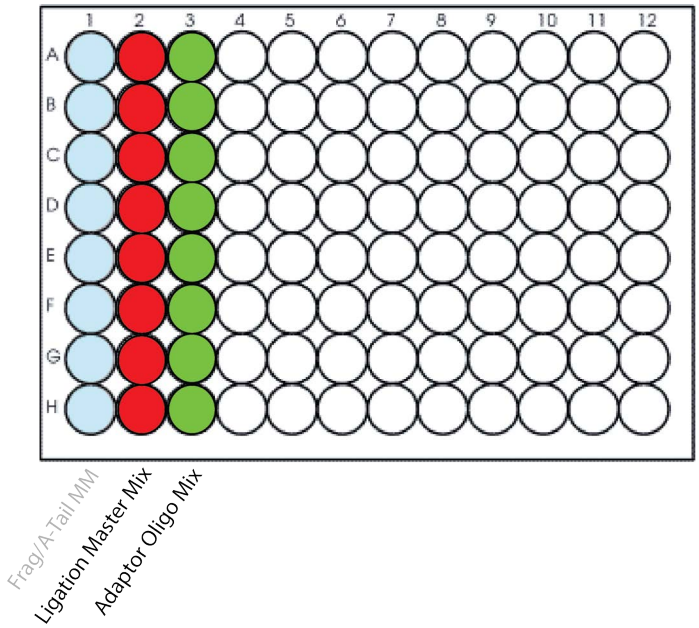


Figure 7 Configuration of the **Agilent Deep Well** master mix source plate for runset AdapLig_Bead_Cleanup_SS_Max_runset. The master mix dispensed into column 1 during a protocol is shown in light shading.

2 Seal the reagent source plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

3 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles. Keep the master mix source plate on ice.

NOTE

The presence of bubbles in source plate solutions may cause inaccurate volume transfer by the Bravo liquid handling platform. Ensure that the source plate is sealed and centrifuged prior to use in a run.

Prepare the purification reagents for AdapLig_Bead_Cleanup_SS_Max runset

1 Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water.

Make sure that the water in the reservoir does not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.

At the end of the automation protocol, retain this reservoir for use in the Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol.

2 Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.

Load the NGS Workstation for AdapLig_Bead_Cleanup_SS_Max runset

1 Load the Labware MiniHub according to **Table 30**, using the plate orientations shown in **Figure 6** on page 59.

Table 30 Initial MiniHub configuration for AdapLig_Bead_Cleanup_SS_Max_runset

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Aliquoted purification beads in Eppendorf twin.tec plate from **page 46** (60 µL of beads/well)	—	—	—
Shelf 4	Empty Eppendorf twin.tec plate	—	—	—
Shelf 3	—	—	—	—
Shelf 2	New tip box (or box from protocol EnzFrag_ERA_SS_Max)	Nuclease-free water reservoir from **page 64**	—	—
Shelf 1 (Bottom)	Empty tip box (or box from protocol EnzFrag_ERA_SS_Max)	70% ethanol reservoir from **page 64**	—	Empty tip box

2 Load the Bravo deck according to **Table 31**.

Table 31 Initial Bravo deck configuration for AdapLig_Bead_Cleanup_SS_Max_runset

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	PCR plate of fragmented and dA-tailed samples transferred from the thermal cycler; seated in red insert (PCR plate type must be specified on setup form under Parameter 3)
5	Empty Eppendorf twin.tec plate
6	Master Mix source plate prepared on **page 63**, unsealed
9	Empty Eppendorf twin.tec plate

3 Load the BenchCel Microplate Handling Workstation according to **Table 32**.

Table 32 Initial BenchCel configuration for AdapLig_Bead_Cleanup_SS_Max_runset

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip boxes	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	3 Tip boxes	—	—	—
12	6 Tip boxes	—	—	—

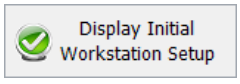
Run VWorks AdapLig_Bead_Cleanup_SS_Max_runset

1 On the SureSelect setup form, under **Select protocol to run**, select **AdapLig_Bead_Cleanup_SS_Max_runset**.

2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

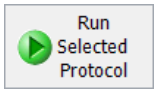
3 Under **Select plate type for thermal cycling**, select the specific type of PCR plate used at position 4 of the Bravo deck.

4 Click **Display Initial Workstation Setup**.

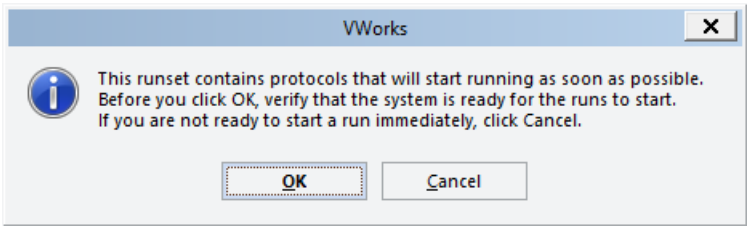


5 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

6 When verification is complete, click **Run Selected Protocol**.



7 When ready to begin the run, click **OK** in the following window.



Running the AdapLig_Bead_Cleanup_SS_Max_runset takes approximately 1 hour. Once complete, the purified, adaptor-ligated DNA samples are located in the Eppendorf plate at position 7 of the Bravo deck.

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

Step 3. Amplify adaptor-ligated libraries

In this step, the NGS Workstation completes the liquid handling steps for setting up the amplification and dual-indexing reactions with the adaptor-ligated DNA samples using the Pre-CapPCR_SS_Max automation protocol. After the NGS Workstation completes the liquid-handling steps, you transfer the PCR plate to a thermal cycler for amplification.

This step of the workflow uses the components listed in **Table 33**. Prepare each component as directed.

Table 33 Reagents thawed before use in Pre-CapPCR_SS_Max

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect Max Enzymatic Fragmentation Library Preparation Module, stored at –20°C	Amplification Master Mix (bottle)	Thaw on ice then keep on ice. Mix thoroughly by inversion at least 5X. **Do not vortex**.	**page 70**
SureSelect Max Adaptors and UDI Primers Kit for ILM, stored at –20°C	SureSelect Max UDI Primers for ILM (select from the following): Index Pairs 1-96 (orange plate) Index Pairs 97-192 (blue plate) Index Pairs 193-288 (green plate) Index Pairs 289-384 (red plate)	Thaw index plate on ice then keep on ice, vortex to mix.	**page 67**

CAUTION

To avoid cross-contaminating libraries, set up PCR master mixes in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

The SureSelect Max UDI Primers are provided in single-use aliquots. To avoid cross-contamination of libraries, do not retain and re-use any residual volume in the plate for subsequent experiments.

Prepare the plate of SureSelect Max UDI Primer Pairs (manually or using the automated protocol)

Prepare the UDI Primer Pairs plate manually

1 Using a multichannel pipette, transfer 5 µL of each SureSelect Max UDI Primers pair from the 96-well plate in which the primers are provided into the PCR plate to be used for the pre-capture PCR thermal cycling. Make sure to maintain the same well location for each primer pair when transferring to the PCR plate. Keep the PCR plate on ice.

The PCR plate containing the primers is loaded onto the Bravo deck in **step 2** on **page 71** for the Pre-CapPCR_SS_Max protocol.

Prepare the UDI Primer Pairs plate using automation protocol Index_Aliquot

1 Clear the Labware MiniHub and BenchCel of all plates and tip boxes.

2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.

3 Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.

4 Load the Bravo deck according to **Table 34**.

Table 34 Initial Bravo deck configuration for Index_Aliquot protocol

Location	Content
2	New tip box
4	SureSelect Max UDI Primers for ILM (in provided 96-well plate; unsealed)
6	Empty PCR plate to be used for the pre-capture PCR thermal cycling; seated in red insert (PCR plate type must be specified on setup form under step 3)

5 In the VWorks software, open the Utility form. See **"Accessing the Supplemental VWorks Forms"** on page 30.

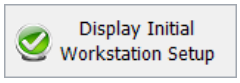
6 Under **Select protocol to run**, select **Index_Aliquot**.

7 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

8 Under **Select plate type for thermal cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

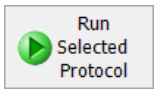
9 Next to **Select index start column**, select the first column number on the plate of SureSelect Max UDI Primers from which to start aliquoting. If you are processing 12 columns of samples, select column 1 as the start column.

10 Click **Display Initial Workstation Setup**.



11 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

12 When verification is complete, click **Run Selected Protocol**.



Running the Index_Aliquot protocol takes approximately 5 minutes. Once the protocol is complete, remove the PCR plate containing the SureSelect Max UDI Primers from position 6, seal the plate, and keep on ice as you continue to the next section (**"Load the NGS Workstation for Pre-CapPCR_SS_Max protocol"**).

Prepare the NGS Workstation for Pre-CapPCR_SS_Max protocol

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
5. Place a red PCR plate insert at Bravo deck position 6.

Pre-program the thermal cycler for pre-capture PCR

1. Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 35**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the automation run.

Table 35 Pre-Capture PCR Thermal Cycler Program (50 µL)

Segment	Number of Cycles	Temperature	Time
1	1	98°C	45 seconds
2	7 to 14, based on input DNA quality and quantity (see **Table 36**)	98°C	15 seconds
		60°C	30 seconds
		72°C	30 seconds
3	1	72°C	1 minute
4	1	4°C	Hold

Table 36 Pre-capture PCR cycle number recommendations

Quality of Input DNA	Quantity of Input DNA	Cycles
Intact DNA from fresh sample	200 ng	7 cycles
	100 ng	8 cycles
	50 ng	9 cycles
	10 ng	10 cycles
FFPE sample DNA	100 to 200 ng*	11 cycles
	50 ng*	12 cycles
	10 ng*	14 cycles

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

Prepare the Amplification Master Mix source plate for Pre-CapPCR_SS_Max protocol

1 Using the same **Agilent Deep Well** master mix source plate that was used for the AdapLig_Bead_Cleanup_SS_Max_runset, add the volume of Amplification Master Mix indicated in **Table 37** to all wells of column 4 of the source plate. The final configuration of the source plate is shown in **Figure 8**.

Table 37 Preparation of the master mix source plate for Pre-CapPCR_SS_Max protocol

Reagent	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Amplification Master Mix	Column 4 (A4-H4)	35.0 µL	60.0 µL	83.5 µL	110.0 µL	160.0 µL	310.0 µL

CAUTION Make sure to add the Amplification Master Mix to column 4 of the source plate.

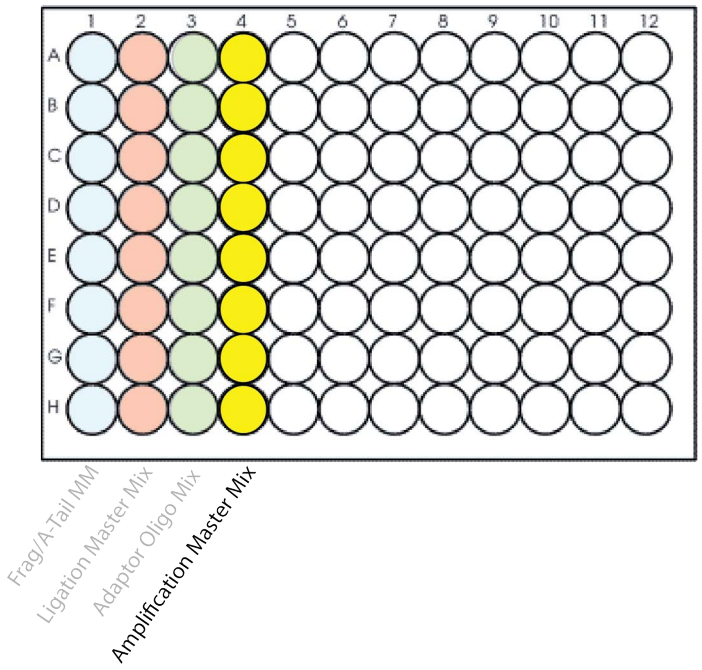


Figure 8 Configuration of the **Agilent Deep Well** Amplification Master Mix source plate for protocol Pre-CapPCR_SS_Max. Master mixes dispensed during previous protocols are shown in light shading.

2 Seal the master mix source plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

3 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

NOTE The presence of bubbles in source plate solutions may cause inaccurate volume transfer by the Bravo liquid handling platform. Ensure that the source plate is sealed and centrifuged prior to use in a run.

Load the NGS Workstation for Pre-CapPCR_SS_Max protocol

1 Load the Labware MiniHub according to **Table 38**, using the plate orientations shown in **Figure 6** on page 59.

Table 38 Initial MiniHub configuration for Pre-CapPCR_SS_Max protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	—	—	—	—
Shelf 1 (Bottom)	—	—	—	Empty tip box

2 Load the Bravo deck according to **Table 39**.

Table 39 Initial Bravo deck configuration for Pre-CapPCR_SS_Max protocol

Location	Content
6	SureSelect Max UDI Primers in PCR plate; seated in red insert (PCR plate type must be specified on setup form under step 3)
7	Adaptor-ligated DNA samples in Eppendorf twin.tec plate
8	Empty tip box
9	Amplification Master Mix source plate containing Amplification Master Mix in column 4 (unsealed)

3 Load the BenchCel Microplate Handling Workstation according to **Table 40**.

Table 40 Initial BenchCel configuration for Pre-CapPCR_SS_Max protocol

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	2 Tip boxes	—	—	—
2	2 Tip boxes	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	2 Tip boxes	—	—	—
12	2 Tip boxes	—	—	—

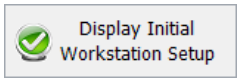
Run VWorks Pre-CapPCR_SS_Max protocol

1 On the SureSelect setup form, under **Select protocol to run**, select the **Pre-CapPCR_SS_Max** protocol.

2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

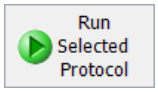
3 Under **Select plate type for thermal cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

4 Click **Display Initial Workstation Setup**.



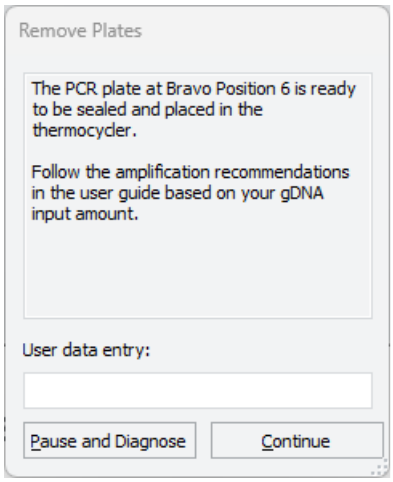
5 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

6 When verification is complete, click **Run Selected Protocol**.



Running the Pre-CapPCR_SS_Max protocol takes approximately 15 minutes. Once complete, the PCR-ready samples, containing prepped DNA and Amplification Master Mix, are located in the PCR plate at position 6 of the Bravo deck.

7 When you see the following prompt, remove the PCR plate from position 6 of the Bravo deck, then click **Continue**. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 seconds.



8 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

9 Before adding the samples to the pre-programmed thermal cycler, bring the temperature of the thermal block to 98°C by resuming the thermal cycler program in **Table 35**. Once the cycler has reached 98°C, immediately place the sample plate in the thermal block and close the lid.

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

10 When the PCR amplification program is complete, spin the plate briefly then keep on ice until it is loaded on the Bravo deck for the next protocol.

Step 4. Purify amplified DNA using beads

In this step, the Agilent NGS Workstation transfers amplified DNA to an Eppendorf twin.tec plate containing SureSelect Max Purification Beads and then collects and washes the bead-bound DNA. Use the automated protocol that is suitable for your workflow:

- Protocol **Bead_Cleanup_SS_Max (Pre-Cap PCR - SinglePlex)**: For the post-capture pooling workflow (i.e., pooling samples after hybridization with the Probe)
- Protocol **Bead_Cleanup_SS_Max (Pre-Cap PCR - MultiPlex)**: For the pre-capture pooling workflow (i.e., pooling samples prior to hybridization with the Probe)

This step uses the aliquoted plate of SureSelect Max Purification Beads that was prepared on **page 48**.

Prepare the NGS Workstation for Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol

1. Set aside the Agilent Deep Well source plate located at position 9 of the Bravo deck for later use in the TS_D1000 protocol (if you plan to analyze the pre-capture libraries on the Agilent 4200 TapeStation). Otherwise, clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Place a red PCR plate insert at Bravo deck position 4.

Prepare the purification reagents for Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol

1. Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water. Use the same Agilent shallow well reservoir that was used in the AdapLig_Bead_Cleanup_SS_Max runset.

   Make sure that the water in the reservoir does not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.
2. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.

Load the NGS Workstation for Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol

1 Load the Labware MiniHub according to **Table 41**, using the plate orientations shown in **Figure 6** on page 59.

Table 41 Initial MiniHub configuration for Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Aliquoted purification beads in Eppendorf twin.tec plate from **page 48** (50 µL of beads/well)	—	—	—
Shelf 4	Empty Eppendorf twin.tec Plate	—	—	—
Shelf 3	—	—	—	—
Shelf 2	—	Nuclease-free water reservoir from **page 74**		—
Shelf 1 (Bottom)	—	70% ethanol reservoir from **page 74**	—	Empty tip box

2 Load the Bravo deck according to **Table 42**.

Table 42 Initial Bravo deck configuration for Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	Amplified DNA libraries in unsealed PCR plate; seated in red insert (PCR plate type must be specified on setup form under step 3)

3 Load the BenchCel Microplate Handling Workstation according to **Table 43**.

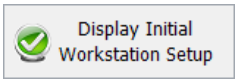
Table 43 Initial BenchCel configuration for Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	3 Tip boxes	—	—	—
12	5 Tip boxes	—	—	—

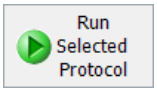
Run VWorks protocol Bead_Cleanup_SS_Max (Pre-Cap PCR)

1. On the SureSelect setup form, under **Select protocol to run**, select one of the Bead_Cleanup_SS_Max (Pre-Cap PCR) protocols based on your workflow of choice.
   - If you are using the post-capture pooling workflow (i.e., pooling samples after hybridization with the Probe) then select the protocol **Bead_Cleanup_SS_Max (Pre-Cap PCR - SinglePlex)**.
   - If you are using the pre-capture pooling workflow (i.e., pooling samples prior to hybridization with the Probe) then select the protocol **Bead_Cleanup_SS_Max (Pre-Cap PCR - MultiPlex)**.

   Selecting the correct option is important for the downstream hybridization protocol.
2. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
3. Under **Select plate type for thermal cycling**, select the specific type of PCR plate containing the amplified libraries at position 4.
4. Click **Display Initial Workstation Setup**.



5. Verify that the NGS Workstation has been set up as displayed at the bottom of the form.
6. When verification is complete, click **Run Selected Protocol**.



The purification protocol takes approximately 45 minutes. When complete, the purified DNA libraries are in the Eppendorf plate located on Bravo deck position 7.

Step 5. Assess Library DNA quantity and quality (optional)

CAUTION Quality assessment of the prepared libraries is optional, but quantification is required for hybridization workflows using library normalization, including pre-capture pooling.

Sample analysis can be completed using one of two options.

- Option 1: Prepare the analytical assay plate using automation (protocol TS_D1000) and perform analysis on Agilent 4200 TapeStation. See **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape"** on page 77.
- Option 2: Prepare the analytical samples manually and perform analysis on Agilent 4200 or 4150 TapeStation or Agilent 5200 Fragment Analyzer. See **"Option 2: Analysis using an equivalent platform (non-automated)"** on page 82.

Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape

This section describes the use of automation protocol TS_D1000 to prepare the D1000 assay sample plate by combining 2 µL of each DNA sample with 6 µL of D1000 sample buffer. Afterward, you transfer the prepared assay plate to the 4200 TapeStation instrument for analysis. For more information to do this step, see the Agilent D1000 Assay Quick Guide for 4200 TapeStation System.

Allow the reagents to equilibrate to room temperature for 30 minutes prior to use.

Prepare the NGS Workstation for TS_D1000 protocol

1. Clear the Labware MiniHub and BenchCel of plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
3. Turn off the ThermoCube device (see **page 24**) to restore position 9 of the Bravo deck to room temperature.
4. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.

Prepare the Sample Buffer source plate for TS_D1000 protocol

5. Using the same **Agilent Deep Well** master mix source plate that was used for the Pre-CapPCR_SS_Max protocol, prepare the Sample Buffer source plate at room temperature. Add the volume of D1000 Sample Buffer indicated in **Table 44** to each well of column 5 of the plate.

Table 44 Preparation of the Sample Buffer source plate for TS_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
D1000 Sample Buffer	Column 5 (A5-H5)	11 µL	17 µL	23 µL	29 µL	42 µL	80 µL

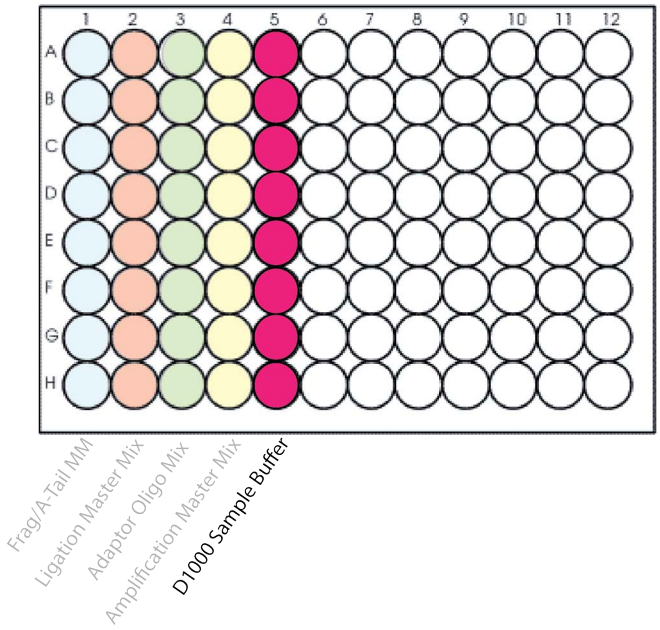


Figure 9 Configuration of the **Agilent Deep Well** source plate for protocol TS_D1000. Master mixes dispensed during previous protocols are shown in light shading.

Load the NGS Workstation

6 Load the Labware MiniHub according to **Table 45**, using the plate orientations shown in **Figure 6** on page 59.

Table 45 Initial MiniHub configuration for TS_D1000 protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	New tip box	—	—	—
Shelf 1 (Bottom)	Empty tip box	—	—	Empty tip box

7 Load the Bravo deck according to **Table 46**.

Table 46 Initial Bravo deck configuration for TS_D1000 protocol

Location	Content
4	Amplified pre-capture libraries in Eppendorf twin.tec plate (unsealed)
6	Empty Eppendorf twin.tec plate
9	Source plate containing D1000 Sample Buffer in Column 5

CAUTION

To prevent damage to the Agilent 4200 TapeStation instrument and the NGS Workstation, use only the specified Eppendorf twin.tec plates (Eppendorf p/n 951020401 or 951020619) for automated assay plate preparation.

The Agilent 2200 TapeStation system does not support use of the Eppendorf twin.tec plates. Do not load sample plates prepared using the automated protocol in the 2200 TapeStation instrument.

8 Load the BenchCel Microplate Handling Workstation according to **Table 47**.

Table 47 Initial BenchCel configuration for TS_D1000 protocol

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

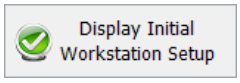
Run VWorks protocol TS_D1000

9 In the VWorks software, open the Utility form. See **"Accessing the Supplemental VWorks Forms"** on page 30.

10 Under **Select protocol to run**, select **TS_D1000**.

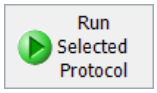
11 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

12 Click **Display Initial Workstation Setup**.



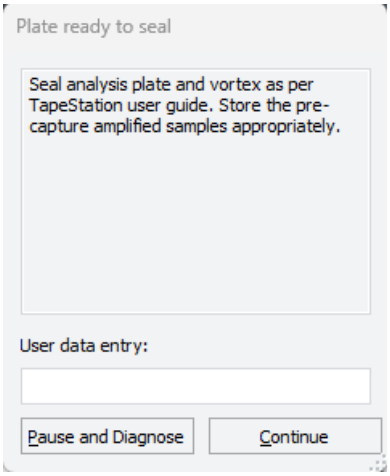
13 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

14 When verification is complete, click **Run Selected Protocol**.



Running the TS_D1000 protocol takes approximately 15 minutes. Once removal of analytical samples is complete, remove the primary DNA library sample plate from position 4, seal the plate, and keep on ice until the samples are used for hybridization set up on **page 83**.

15 When prompted by VWorks as shown below, remove the Eppendorf twin.tec plate containing the analytical samples from position 6 of the Bravo deck, then click **Continue**. Seal the D1000 assay plate with a foil seal and vortex the sealed plate as directed in the Agilent D1000 Assay Quick Guide for 4200 TapeStation System.



CAUTION

To prevent damage to the Agilent 4200 TapeStation instrument, use only the specified 96-well plate foil seals (Agilent p/n 5067-5154).

For accurate quantitation, make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 1 minute, then spin briefly to collect the liquid.

Run the D1000 Assay and analyze the data

16 Load the analytical sample plate, the D1000 ScreenTape, and loading tips into the TapeStation instrument as instructed in the D1000 Assay Quick Guide. Start the run.

17 Using the 150 bp to 1000 bp region of the electropherogram, verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 48** for guidelines). Representative electropherograms are shown in **Figure 10** and **Figure 11** to illustrate typical results

Observation of a low molecular weight peak, in addition to the expected library fragment peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, as shown in **Figure 10**. See Troubleshooting information on **page 178** for additional considerations.

Table 48 Pre-capture library qualification guidelines

Input type	NGS read length used to select fragmentation duration	Expected average fragment size (150–1000 bp region)
Intact DNA	2 × 100 reads	250 to 450 bp
	2 × 150 reads	280 to 480 bp
FFPE DNA	2 × 100 reads OR 2 × 150 reads	250 to 360 bp

18 Determine the concentration of the library DNA by integrating under the peak.

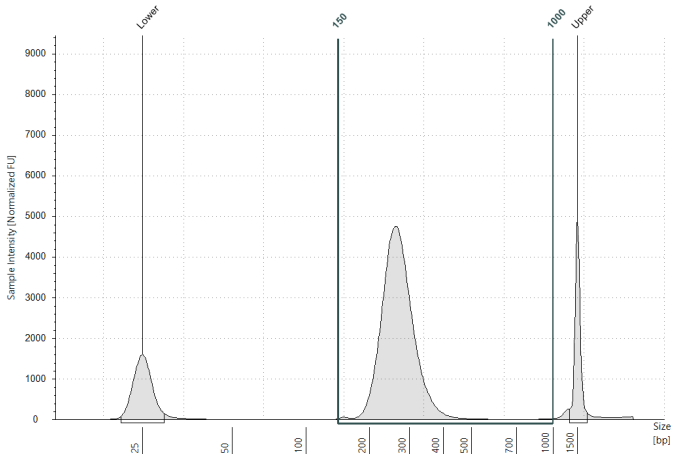


Figure 10 Pre-capture library prepared from an FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

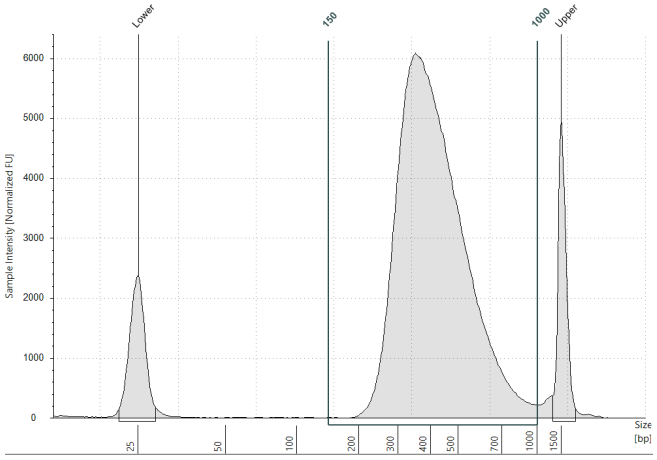


Figure 11 Pre-capture library prepared from a high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

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

Option 2: Analysis using an equivalent platform (non-automated)

Using manual preparation of the analytical sample plate, you can analyze the DNA samples on an Agilent 5200 Fragment Analyzer or Agilent 4200/4150 TapeStation. Electropherograms obtained using one of these other analysis platforms are expected to show fragment size profiles similar to those shown for the Agilent 4200 TapeStation (see **Figure 10** and **Figure 11**). Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 48** for guidelines). **Table 49** includes links to assay instruction.

Table 49 Pre-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	D1000 ScreenTape	**Agilent D1000 Assay Quick Guide**	1 µL of a five-fold dilution
Agilent 5200, 5300, or 5400 Fragment Analyzer system	NGS Fragment Kit (1-6000 bp)	**Agilent NGS Fragment Kit (1-6000 bp) Guide**	2 µL of a five-fold dilution

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.

6 Hybridization Preparation

This chapter describes the steps to prepare the plate of DNA libraries or DNA library pools that will be used to set up the hybridization reactions.

Preparation of the DNA libraries for hybridization differs depending on the sample pooling strategy.

- If you are pooling samples after hybridization to the Probe, then follow the instructions in **"Option 1. Prepare DNA for Single-Plex Hybridization"** on page 84.
- If you are pooling samples prior to hybridization to the Probe, then follow the instructions in **"Option 2. Prepare DNA for Multi-Plex Hybridization"** on page 85.

Option 1. Prepare DNA for Single-Plex Hybridization

If you are using the post-capture pooling workflow, the Eppendorf twin.tec plate containing purified DNA libraries that was prepared during the Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol is ready for the Hybridization automation protocol. No further sample preparation is required and you can proceed directly to the appropriate Hybridization chapter:

- **Chapter 7**, "Hybridization (Fast)," starting on page 95
- **Chapter 8**, "Hybridization (Overnight)," starting on page 107

NOTE

For the Fast Hybridization workflow, if you prefer to use a specific DNA quantity in the hybridization reactions, you can prepare a sample plate that contains the desired quantity of DNA. See**"Using the Aliquot_Libraries automated protocol"** on page 160 for instructions.

Option 2. Prepare DNA for Multi-Plex Hybridization

Follow the steps in this section if you are using the pre-capture pooling workflow.

Step 1. Plan sample pooling setup

Before pooling the indexed gDNA samples using the Pooling protocol, plan which samples to pool together and the configuration of the destination plate into which the NGS Workstation pools the samples.

Plan pooling run parameters

The hybridization reaction requires 4000 ng of indexed gDNA pool. The pool contains equal amounts of either 8 or 16 individual indexed gDNA libraries. See **Table 50** for the recommended pool composition based on your SureSelect Probe.

Table 50 Pre-capture pooling of indexed DNA libraries

Probe	Number of DNA libraries per pool	Amount of total DNA per pool (Amount of DNA pool per hybridization reaction)*	Amount of each DNA library in pool	Maximum DNA concentration for pool
SureSelect XT HS PreCap Human All Exon V7 or V8 (including V8+UTR/V8+NCV) or SureSelect XT HS Clinical Research Exome V4	8	8000 ng (4000 ng/hybridization)	1000 ng (500 ng/hybridization)	500 ng/µL
SureSelect XT HS PreCap Custom Probes	16	8000 ng (4000 ng/hybridization)	500 ng (250 ng/hybridization)	250 ng/µL

* Where possible, indexed DNA pools are prepared containing a total DNA amount that is enough for two hybridization reactions. For some indexed DNA pools, the initial library pool will contain enough total DNA for more than two hybridization reactions.

Before setting up the pooling run, you must determine the total amount of DNA to pool and the volumes of the pools based on the starting concentrations of the DNA samples to be pooled.

Accurate normalization of pools requires a minimum pipetting volume of 2 µL for each sample. Maximum DNA concentration values for a pool containing >2 µL of each sample are shown in **Table 50**, above. When higher-concentration DNA samples are included in the pooling run, the DNA pool amount must be adjusted as described below.

- Check the DNA concentration of each sample in the set of source plates to be pooled to a single destination plate to determine the appropriate amount of DNA per pool.
  - If all samples contain DNA at concentrations below the maximum DNA concentration shown in **Table 50**, then prepare pools with 8000 ng of DNA.

- If at least one of the samples is above the maximum DNA concentration shown in **Table 50**, then you need to calculate the appropriate DNA pool amount. First, identify the most concentrated DNA sample and calculate the amount of DNA contained in 2 µL of that sample. This becomes the amount of each DNA sample used for pooling in the run. For example, if the highest DNA sample concentration is 600 ng/µL, then the final DNA pool will contain 1200 ng of each indexed DNA. Next, determine the total amount of DNA per pool, based on the Probe size. Continuing with the same example, an All Exon V8 capture pool would contain 8 × 1200 ng, or 9600 ng DNA.

Plan configuration of destination plate for pooled indexed samples

The indexed gDNA samples should be pooled into the destination plate using a pooled sample configuration appropriate for the subsequent hybridization run. Use the following plate configuration considerations for pooling gDNA samples for automated hybridization and capture runs:

- When using a single Probe for all wells on the plate, fill the plate column-wise in well order A1 to H1, then A2 to H2, ending with A12 to H12.
- When using multiple Probes, configure the plate such that all gDNA library pools to be hybridized to a particular Probe are positioned in appropriate rows. When preparing for the Hyb_SS_Max protocol, place samples to be enriched using the same Probe in the same row.
- Each 96-reaction library preparation run produces 6 or 12 gDNA pools. For greatest efficiency of reagent use, gDNA pools from multiple library preparation runs may be placed on the same destination plate for hybridization.

Step 2. Pool indexed DNA samples for hybridization

In this step, the NGS Workstation pools the prepped indexed gDNA samples before hybridization to the Probe. This workflow step is set up using the SureSelect Max DNA Pooling and Normalization VWorks Form.

Prepare CSV files for pooling and normalization

Before starting the Pooling automation protocol, you must create comma-separated value (CSV) files containing instructions including the specific wells to be pooled and the concentration of each sample. From this data, the NGS Workstation calculates the volume of each sample required to prepare each concentration-normalized pool for the hybridization step.

See **Figure 12** for required CSV file content. Pooling and normalization CSV file templates are provided in the following directory:

C:\VWorks Workspace\NGS Option B\Agilent_SS_Max_DNA_B1.1.1.2\Pooling and Normalization Templates

Select the appropriate set of templates from the directory based on the intended pool composition (8 or 16 prepared samples) and on the number of source plates to be consolidated in the run to prepare the single destination plate containing the hybridization samples. For example, for 8-library pools, use the template Pool8_01_SourcePlate.csv for the first DNA source plate, continuing with additional Pool8_0X_SourcePlate.csv files for additional DNA source plates.

1 Copy and rename the appropriate set of CSV file templates for the run. Make sure to retain the header text used in the template files, without introducing spaces or other new characters.

   If processing a partial plate of prepped gDNA samples, delete the rows corresponding to the WellIDs of the empty wells on the plate.

	A	B	C
1	Well ID	PreCap Amplified pond concentrations (ng/ul)	Target WellID
2	A1	52.79	A1
3	B1	49.21	A1
4	C1	38.73	A1
5	D1	43.56	A1
6	E1	39.7	A1
7	F1	45.33	A1
8	G1	53.38	A1
9	H1	48.91	A1
10	A2	40.74	B1
11	B2	37.22	B1
12	C2	42.03	B1

Figure 12 Sample pooling and normalization CSV file content

2 In each CSV file, edit the information for each DNA sample (well ID) as follows:

- In the **PreCap Amplified pond concentrations** field, enter the concentration (in ng/μL) determined on **page 77** for each indexed DNA sample.
- In the **Target WellID** field, enter the well position of the pool in which the indexed DNA sample should be included for the destination plate. See the guidelines on **page 85** for hybridization sample pool placement considerations.

Prepare the NGS Workstation for the Pooling protocol

3 Clear the Labware MiniHub and BenchCel of all plates and tip boxes.

4 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.

5 Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.

Load the NGS Workstation for the Pooling protocol

6 If you will be loading the source plates on the MiniHub (rather than loading them manually when prompted), then load the Labware MiniHub according to **Table 51**, using the plate orientations shown in **Figure 6** on page 59. If you are using fewer than 8 source plates, then leave the MiniHub positions for the unused source plates empty.

Table 51 Initial MiniHub configuration for Pooling protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Source plate 5	—	—	—
Shelf 4	Source plate 4	—	—	—
Shelf 3	Source plate 3	Source plate 8	—	—
Shelf 2	Source plate 2	Source plate 7	—	—
Shelf 1 (Bottom)	Source plate 1	Source plate 6	—	—

7 Load the Bravo deck according to **Table 52**.

Table 52 Initial Bravo deck configuration for Pooling protocol

Location	Content
5	Destination plate (unsealed)
6	Empty tip box

8 Load the BenchCel Microplate Handling Workstation according to **Table 53**.

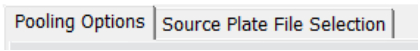
Table 53 Initial BenchCel configuration for Pooling protocol

No. of Source Plates	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	2 Tip boxes	—	—	—
3	3 Tip boxes	—	—	—
4	4 Tip boxes	—	—	—
5	5 Tip boxes	—	—	—
6	6 Tip boxes	—	—	—
7	7 Tip boxes	—	—	—
8	8 Tip boxes	—	—	—

Run the VWorks Pooling protocol

9 In the VWorks software, open the Pooling and Normalization form. See **"Accessing the Supplemental VWorks Forms"** on page 30.

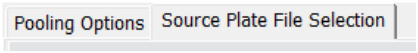
10 On the Pooling Options tab of the form, set up the run parameters described below.



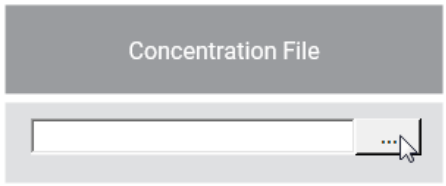
- Next to **Number of Indexes to Pool**, select 8 or 16 (see **Table 50** on page 85 for guidelines).
- Next to **Pooled DNA Quantity [ng] (2 Hybridizations)**, enter the required total amount of DNA in the pool.

  The default required amount is 8000 ng, which is sufficient for two hybridization reactions.
- Next to **Number of Source Plates (1-8)**, select the number of indexed DNA source plates to be provided for sample pooling. If >8 plates will be used to create a single hybridization sample plate, run the Pooling protocol in sets of 8 source plates.
- Next to **Load Sources**, specify whether the indexed DNA source plate(s) were loaded to the MiniHub or if they will be loaded manually.
- Next to **Source Plates Enter Sealed**, specify whether the indexed DNA source plate(s) will be sealed at start of run. If you select **Yes**, you will be prompted during the Pooling protocol to remove each plate seal.
- Next to **Destination Plate ID/Barcode**, enter the name or barcode of the destination plate into the field provided.

11 On the Source Plate File Selection tab of the form, select the CSV files that provide sample position and concentration data for each source plate. This tab lists the information for up to 8 source plates.

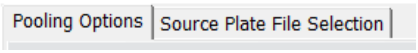


a In the **Concentration File** field, click the "**...**" browse button to specify the location of the CSV file for each source plate.



b In the **Plate ID/Barcode** field, enter the name or barcode of the source plate.

12 When finished selecting the CSV files for the source plates, return to the Pooling Options tab.



13 Click **Display Initial Workstation Setup**.

14 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

15 When verification is complete, click **Run Pooling Protocol**.

CAUTION When more than one indexed DNA source plate is used in the run, and you selected to load the source plate manually and/or the source plates are sealed, an operator must remain present throughout the Pooling protocol to respond to NGS Workstation prompts.

Running the Pooling protocol takes approximately one hour per indexed DNA source plate. Once complete, the destination sample plate, containing indexed DNA pools, is located at position 5 of the Bravo deck.

16 Remove the destination plate from Bravo deck position 5.

17 Seal the Eppendorf twin.tec plate containing the indexed DNA pool samples using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep on ice.

Step 3. Adjust final concentration of pooled DNA

Prior to hybridization with the Probe, use the Aliquot_Water and Bead_Cleanup_SS_Max (Concentration of Pool) automation protocols to concentrate each DNA library pool to a volume sufficient for two hybridization reactions. First, the Aliquot_Water protocol adds enough water to each DNA library pool to bring the volume to 100 µL. Then, the Bead_Cleanup_SS_Max (Concentration of Pool) protocol uses SureSelect Max Purification Beads to purify the DNA library pools, eluting the DNA in a volume sufficient for two hybridization reactions (8 µL for overnight hyb or 24 µL for fast hyb).

This step uses the aliquoted plate of SureSelect Max Purification Beads that was prepared on **page 50**.

Prepare CSV file for normalizing sample volumes to 100 µL

1 Create a CSV (comma separated value) file with the headers shown in **Figure 13**. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in CSV format. The file must include rows for all 96 wells of the plate.

2 Enter the information requested in the header for each DNA sample.

- In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
- In the SourceWell and DestinationWell fields, enter each well position for the plate. SourceWell and DestinationWell field contents must be identical for a given sample.
- In the Volume field, enter the volume (in µL) of water to be added to the sample in the indicated well position in order to bring the total well volume to 100 µL. For all empty wells on the plate, delete the corresponding rows in the CSV file.

	A	B	C	D
1	SourceBC	SourceWell	DestinationWell	Volume
2	abc	A1	A1	5.5
3	abc	B1	B1	5.2
4	abc	C1	C1	11
5	abc	D1	D1	5.9
6	abc	E1	E1	17.5
7	abc	F1	F1	5.5
8	abc	G1	G1	23
9	abc	H1	H1	5.6
10	abc	A2	A2	5.4

Figure 13 Sample Aliquot_Water CSV file content

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\Agilent_SS_Max_DNA_B1.1.1.2\Aliquot Input File Templates**.

The Aliquot_Water_Template.csv file may be copied and used as a template for creating the CSV file for each Aliquot_Water protocol run. If you are using the sample file as a template for runs with fewer than 12 columns, be sure to delete rows for any unused columns on the plate.

3 Load the CSV file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\Agilent_SS_Max_DNA_B1.1.1.2\Pooling and Normalization Templates**.

Set up and run the Aliquot_Water automation protocol

4 Gently wipe down the Bravo deck with a decontamination wipe.

5 Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water.

Make sure that the water in the reservoir does not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.

At the end of the automation protocol, retain this reservoir for use in the Bead_Cleanup_SS_Max (Concentration of Pool) protocol.

6 Load the Bravo deck according to **Table 54**.

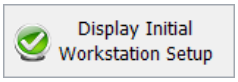
Table 54 Initial Bravo deck configuration for Aliquot_Water protocol

Location	Content
5	Pooled library DNA in Eppendorf twin.tec plate
6	Empty tip box
8	New tip box
9	Nuclease-free water reservoir from **step 5**

7 In the VWorks software, open the Utility form. See **"Accessing the Supplemental VWorks Forms"** on page 30.

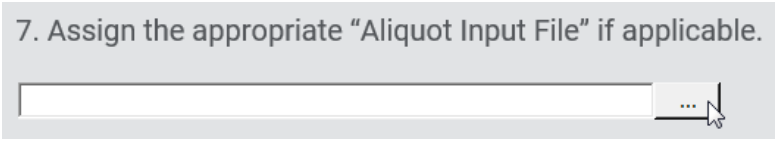
8 Under **Select protocol to run**, select the **Aliquot_Water** protocol.

9 Click **Display Initial Workstation Setup**.



10 Upload the CSV file created on **page 90**.

a Click the "**...**" browse button below **Assign the appropriate "Aliquot Input File" if applicable** to open a directory browser window.

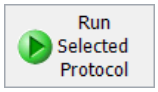


b Browse to the location where you saved the CSV file. Select the file and click **Open**.

The directory browser window closes, returning you to the Utility form. The selected file location is listed in the field.

11 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

12 When verification is complete, click **Run Selected Protocol**.



The water aliquoting protocol takes approximately 1 hour for 96 sample pools. When complete, the DNA sample plate is on Bravo deck position 5.

13 Remove the sample plate from the Bravo deck.

14 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

Set up and run the Bead_Cleanup_SS_Max (Concentration of Pool) protocol

15 Clear the Labware MiniHub and BenchCel of all plates and tip boxes.

16 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.

17 Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water.

Make sure that the water in the reservoirs does not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.

18 Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.

19 Load the Labware MiniHub according to **Table 55**, using the plate orientations shown in **Figure 6** on page 59.

Table 55 Initial MiniHub configuration for Bead_Cleanup_SS_Max (Concentration of Pool) protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Aliquoted purification beads in Agilent deep well plate from **page 50** (180 µL of beads/well)	—	—	—
Shelf 4	Empty Eppendorf twin.tec Plate	—	—	—
Shelf 3	—	—	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 17**		—
Shelf 1 (Bottom)	—	70% ethanol reservoir from **step 18**	—	Empty tip box

20 Load the Bravo deck according to **Table 56**.

Table 56 Initial Bravo deck configuration for Bead_Cleanup_SS_Max (Concentration of Pool) protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	PCR plate containing DNA library pools from the Aliquot_Water protocol; seated in red insert (PCR plate type must be specified on setup form under step 3)

21 Load the BenchCel Microplate Handling Workstation according to **Table 57**.

Table 57 Initial BenchCel configuration for Bead_Cleanup_SS_Max (Concentration of Pool) protocol

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	3 Tip boxes	—	—	—
12	5 Tip boxes	—	—	—

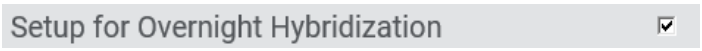
22 On the Utility form, under **Select protocol to run**, select the **Bead_Cleanup_SS_Max (Concentration of Pool)** protocol.

23 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

24 Under **Select plate type for thermal cycling**, select the specific type of PCR plate used at position 4 of the Bravo deck.

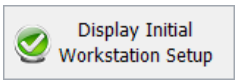
25 Specify which hybridization workflow to use. This selection determines the elution volume of the purified DNA library pools.

- If you will be performing overnight hybridization, mark the check box next to **Setup for Overnight Hybridization**.



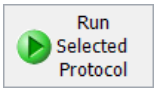
- If you will be performing fast hybridization, make sure this check box is cleared.

26 Click **Display Initial Workstation Setup**.



27 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

28 When verification is complete, click **Run Selected Protocol**.



The purification protocol takes approximately 45 minutes. When complete, the purified DNA library pools are in the Eppendorf plate located on Bravo deck position 7.

29 Proceed to the appropriate Hybridization chapter:

- **Chapter 7**, "Hybridization (Fast)," starting on page 95
- **Chapter 8**, "Hybridization (Overnight)," starting on page 107

7 Hybridization (Fast)

This chapter describes the steps to hybridize the DNA libraries or library pools to the Probe using the fast hybridization method.

The NGS Workstation completes the liquid handling steps to set up the hybridization reactions using the Hyb_SS_Max automation protocol. First, the NGS Workstation combines the samples with the Blocker Mix. You then transfer the sample plate to the thermal cycler for sample denaturation and blocking prior to hybridization. Afterward, you transfer the sample plate back to the NGS Workstation for set up of the hybridization reactions. You then transfer the sample to the thermal cycler again to allow hybridization of the DNA samples to the probe. The hybridization segment of the thermal cycler program is 1 to 2 hours.

NOTE For instructions on the overnight hybridization method, see **Chapter 8**, "Hybridization (Overnight)."

CAUTION The ratio of probe to prepped library is critical for successful capture.

This chapter uses the components listed in **Table 58**. Prepare each component as directed. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 58 Reagents thawed before use in Hyb_SS_Max protocol for fast hybridization

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect Max Blockers and Primers Module for ILM, stored at –20°C	Blocker Mix, ILM (blue cap)	Thaw and keep on ice	page 100
SureSelect Max Target Enrichment Kit Fast Hyb Module Box 2, stored at –20°C	SureSelect RNase Block (purple cap)	Thaw and keep on ice; vortex to mix	page 98
	SureSelect Max Fast Hyb Buffer (bottle)	Thaw and keep at room temperature	page 98
–80°C	SureSelect Probe	Thaw and keep on ice; vortex to mix	page 98

Pre-program the thermal cycler for fast hybridization

1 Pre-program a thermal cycler with the appropriate fast hybridization program.

- **Table 59** – hybridization program for the SureSelect XT HS Human All Exon V8 Probes (V8/V8+UTR/V8+NCV) and SureSelect XT HS Clinical Research Exome V4 Probe
- **Table 60** – hybridization program for all other probes

Set the heated lid to 105°C. Pre-warm the instrument before samples are loaded.

Table 59 Fast hybridization program for the SureSelect XT HS Human All Exon V8/V8+UTR/V8+NCV or Clinical Research Exome V4 Probes (30 µL volume)

Segment	Purpose	Number of Cycles	Temperature	Time
1	Denaturation	1	95°C	5 minutes
2	Blocking	1	65°C	10 minutes
3	Hold for NGS Workstation steps*	1	65°C	Hold
4	Hybridization	60	68°C	1 minute
			37°C	3 seconds
5	Hyb Extension	1	68°C	60 minutes
6	Hold until start of Capture†	1	68°C	Hold (briefly)

* Samples are transferred to the NGS Workstation during this Hold step when prompted by VWorks software.

† Samples are held at 68°C until they are processed in the Capture & Wash automation protocol.

Table 60 Fast hybridization program for *all other SureSelect XT HS probes* (30 µL volume)

Segment	Purpose	Number of Cycles	Temperature	Time
1	Denaturation	1	95°C	5 minutes
2	Blocking	1	65°C	10 minutes
3	Hold for NGS Workstation steps*	1	65°C	Hold
4	Hybridization	60	68°C	1 minute
			37°C	3 seconds
5	Hold until start of Capture†	1	68°C†	Hold (briefly)

* Samples are transferred to the NGS Workstation during this Hold step when prompted by VWorks software.

† Samples are held at 68°C until they are processed in the Capture & Wash automation protocol.

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

NOTE

The protocol may be modified for a 2-day workflow with an overnight pause by making the following changes:

- In the final segment of the thermal cycler program, replace the final brief 68°C Hold step with a 21°C Hold step. The hybridized samples may be held at 21°C for up to 16 hours.
- Do not start the hybrid capture reagent preparation steps while the hybridization thermal cycler program is still running. Instead begin the capture preparation steps on day 2.

Prepare the NGS Workstation for Hyb_SS_Max protocol

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
3. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
4. Place a red PCR plate insert at Bravo deck position 4.

Load the Labware MiniHub and BenchCel for Hyb_SS_Max protocol

NOTE

To avoid prolonged exposure of the master mix source plate to room temperature, load the Labware MiniHub and BenchCel Microplate Handling Workstation prior to preparation of the source plate.

1. Load the Labware MiniHub according to **Table 61**, using the plate orientations shown in **Figure 6** on page 59.

Table 61 Initial MiniHub configuration for Hyb_SS_Max protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	—	—	—	—
Shelf 1 (Bottom)	—	—	—	Empty tip box

2. Load the BenchCel Microplate Handling Workstation according to **Table 62**.

Table 62 Initial BenchCel configuration for Hyb_SS_Max protocol

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	2 Tip boxes	—	—	—
2	2 Tip boxes	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	2 Tip boxes	—	—	—
12	3 Tip boxes	—	—	—

Prepare one or more Fast Hybridization master mixes

1 Prepare the appropriate volume of Fast Hybridization master mix for each of the Probes that will be used for hybridization as indicated in **Table 63** to **Table 66**. Mix by vortexing at medium speed for 15–20 seconds, then spin down briefly. Keep the Fast Hybridization master mix(es) on ice.

NOTE

Each row of the prepped gDNA sample plate may be hybridized to a different Probe. However, Probes of different sizes require different post-capture amplification cycles. Plan experiments such that similar-sized Probes are hybridized on the same plate.

For runs that use a single Probe for all rows of the plate, prepare the master mix as described in Step a (**Table 63** or **Table 64**).

For runs that use different Probes for individual rows, prepare each master mix as described in Step b (**Table 65** or **Table 66**).

a For runs that use a single Probe for all rows, prepare a master mix as described in **Table 63** or **Table 64**, according to the probe design size.

b For runs that use different Probes in individual rows, prepare a master mix for each Probe as listed in **Table 65** or **Table 66**, according to the probe design size. The volumes listed in **Table 65** and **Table 66** are for a single row of sample wells. If a given Probe will be hybridized in multiple rows, multiply each of the values below by the number of rows assigned to that Probe.

Table 63 Preparation of Fast Hybridization master mix for Probes <3 Mb, 8 rows of wells

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	75.3 µL	115.3 µL	144.0 µL	191.9 µL	275.4 µL	533.1 µL
SureSelect RNase Block, undiluted (purple cap)	0.5 µL	8.4 µL	12.8 µL	16.0 µL	21.3 µL	30.6 µL	59.2 µL
SureSelect Max Fast Hyb Buffer (bottle)	6.0 µL	100.4 µL	153.8µL	192.0 µL	255.9 µL	367.2 µL	710.9 µL
Probe (with design <3.0 Mb)	2.0 µL	33.5 µL	51.3 µL	64.0 µL	85.3 µL	122.4 µL	237.0 µL
Total Volume	**13.0 µL**	**217.6 µL**	**333.2 µL**	**416.0 µL**	**554.4 µL**	**795.6 µL**	**1540.2 µL**

Table 64 Preparation of Fast Hybridization master mix for Probes ≥3 Mb, 8 rows of wells, fast hybridization

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	25.1 µL	38.4 µL	48.0 µL	64.0 µL	91.8 µL	177.7 µL
SureSelect RNase Block, undiluted (purple cap)	0.5 µL	8.4 µL	12.8 µL	16.0 µL	21.3 µL	30.6 µL	59.2 µL
SureSelect Max Fast Hyb Buffer (bottle)	6 µL	100.4 µL	153.8 µL	192.0 µL	255.9 µL	367.2 µL	710.9 µL
Probe (with design ≥3.0 Mb)	5 µL	83.7 µL	128.2 µL	160.0 µL	213.2 µL	306.0 µL	592.4 µL
Total Volume	**13.0 µL**	**217.6 µL**	**333.2 µL**	**416.0 µL**	**554.4 µL**	**795.6 µL**	**1540.2 µL**

Table 65 Preparation of Fast Hybridization master mix for Probes <3 Mb, single row of wells, fast hybridization

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	5.8 µL	8.8 µL	11.6 µL	14.9 µL	21.1 µL	40.8 µL
SureSelect RNase Block, undiluted (purple cap)	0.5 µL	0.6 µL	1.0 µL	1.3 µL	1.7 µL	2.3 µL	4.5 µL
SureSelect Max Fast Hyb Buffer (bottle)	6 µL	16.6 µL	25.5 µL	33.4 µL	43.2 µL	60.8 µL	117.8 µL
Probe (with design <3 Mb)	2 µL	2.6 µL	3.9 µL	5.1 µL	6.6 µL	9.4 µL	18.1 µL
Total Volume	**13.0 µL**	**25.6 µL**	**39.2 µL**	**51.4 µL**	**66.4 µL**	**93.6 µL**	**181.2 µL**

Table 66 Preparation of Fast Hybridization master mix for Probes ≥3 Mb, single row of wells, fast hybridization

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	1.9 µL	2.9 µL	3.9 µL	5.0 µL	7.0 µL	13.6 µL
SureSelect RNase Block, undiluted (purple cap)	0.5 µL	0.6 µL	1.0 µL	1.3 µL	1.7 µL	2.3 µL	4.5 µL
SureSelect Max Fast Hyb Buffer (bottle)	6 µL	16.6 µL	25.5 µL	33.4 µL	43.2 µL	60.8 µL	117.8 µL
Probe (with design ≥3 Mb)	5 µL	6.4 µL	9.8 µL	12.8 µL	16.6 µL	23.4 µL	45.3 µL
Total Volume	**13.0 µL**	**25.6 µL**	**39.2 µL**	**51.4 µL**	**66.4 µL**	**93.6 µL**	**181.2 µL**

Prepare the master mix source plate for Hyb_SS_Max protocol

1 Using an **Agilent Deep Well** plate, prepare the hybridization master mix source plate at room temperature, containing the Fast Hybridization master mixes prepared in the previous step and the Blocker Mix. Add the volumes indicated in **Table 67** of each master mix to each well of the indicated column of the plate. When using multiple Probes in a run, add the Fast Hybridization master mix for each probe to the appropriate row(s) of the plate. The final configuration of the master mix source plate is shown in **Figure 14**.

Table 67 Preparation of the master mix source plate for Hyb_SS_Max protocol (fast hybridization)

Master Mix Solution	**Position on Source Plate**	**Volume of master mix added per Well of Agilent Deep Well Source Plate**					
		1-Column Runs	**2-Column Runs**	**3-Column Runs**	**4-Column Runs**	**6-Column Runs**	**12-Column Runs**
Blocker Mix, ILM (blue cap)	Column 1 (A1-H1)	17.0 µL	22.5 µL	21.6 µL	28.8 µL	43.2 µL	83.5 µL
Fast Hybridization master mix	Column 2 (A2-H2)	25.6 µL	39.2 µL	51.4 µL	66.4 µL	93.6 µL	181.2 µL

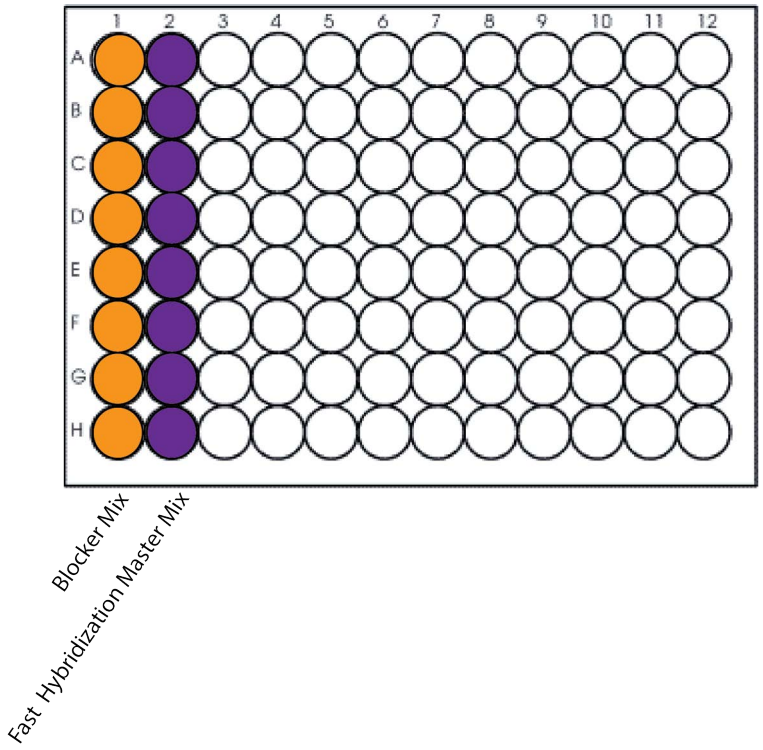


Figure 14 Configuration of the **Agilent Deep Well** master mix source plate for protocol Hyb_SS_Max. Column 2 can contain different Fast Hybridization master mixes in each row.

2 Proceed immediately to loading the Bravo deck, keeping the master mix plate at room temperature only briefly during the loading process.

Load the Bravo deck for Hyb_SS_Max protocol

1 Load the Bravo deck according to **Table 68**.

Table 68 Initial Bravo deck configuration for Hyb_SS_Max protocol

Location	Content
4	Empty PCR plate; seated in red insert (PCR plate type must be specified on setup form under step 3)
5	Empty Eppendorf twin.tec plate
6	Prepared library aliquots or library pools in Eppendorf twin.tec plate (unsealed)
8	Empty tip box
9	Agilent Deep Well Master Mix source plate (unsealed)

Run the VWorks Hyb_SS_Max protocol

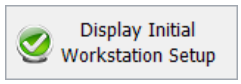
1 On the SureSelect setup form, under **Select protocol to run**, select the **Hyb_SS_Max** protocol.

2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

3 Under **Select plate type for thermal cycling**, select the specific type of PCR plate used at position 4 of the Bravo deck.

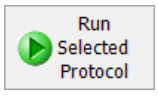
4 Make sure that the check box next to **Setup for Overnight Hybridization** is cleared.

5 Click **Display Initial Workstation Setup**.



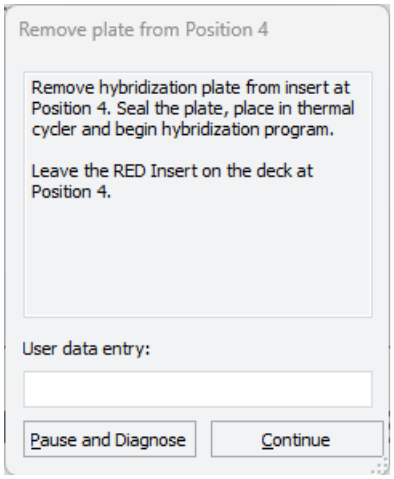
6 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

7 When verification is complete, click **Run Selected Protocol**.



The NGS Workstation combines the prepped gDNA in the wells of the sample plate with the aliquoted Blocker Mix. When this process is complete, you will be prompted to transfer the plate to the thermal cycler for sample denaturation and blocking prior to hybridization.

8 When prompted by VWorks as shown below, remove the PCR plate from position 4 of the Bravo deck, leaving the red insert in place. After removing the sample plate, click **Continue**.



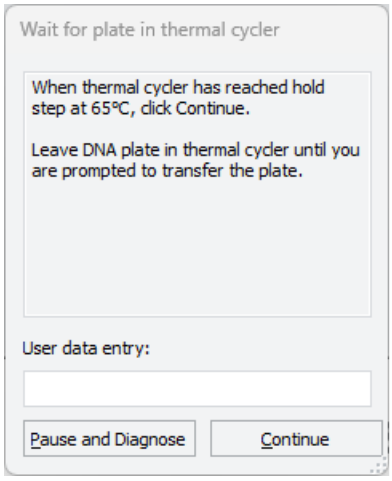
9 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

10 Transfer the sealed plate to a thermal cycler. Initiate the pre-programmed thermal cycler program (**Table 59** on page 96 or **Table 60** on page 96).

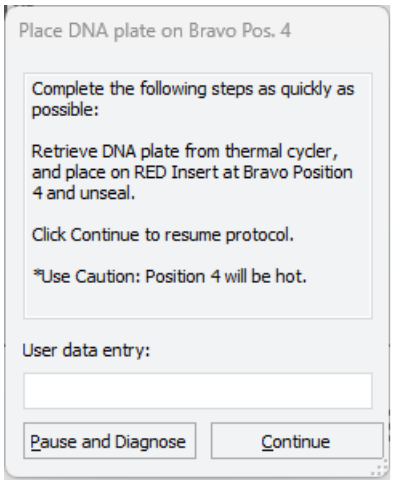
While the sample plate incubates on the thermal cycler, the NGS Workstation aliquots the Fast Hybridization master mix to the Eppendorf twin.tec plate.

CAUTION You must complete **step 11** to **step 15** quickly, and immediately after being prompted by the VWorks software. It is important that sample temperature remains approximately 65°C during transfers between the NGS Workstation and thermal cycler.

11 When the NGS Workstation has finished aliquoting the Fast Hybridization master mix, you will be prompted by VWorks as shown below. When the thermal cycler reaches the 65°C hold step, click **Continue**. Leave the sample plate in the thermal cycler until you are notified to move it.



12 When prompted by VWorks as shown below, quickly remove the sample plate from the thermal cycler, unseal the plate carefully to avoid splashing, and transfer the plate to position 4 of the Bravo deck, seated in the red insert. Click **Continue**.



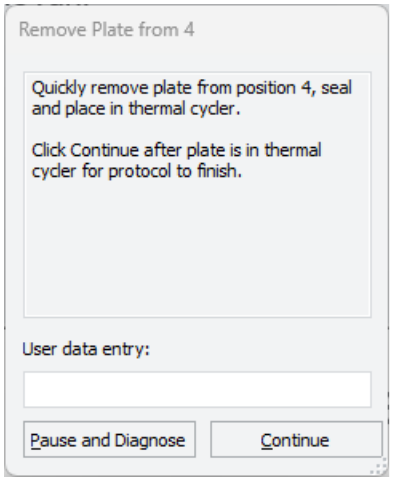
WARNING

Bravo deck position 4 will be hot.

Use caution when handling components that contact heated deck positions.

The NGS Workstation transfers the Fast Hybridization master mix to the wells of the PCR plate that contain the mixture of prepped gDNA samples and blocking agents.

13 When prompted by VWorks as shown below, quickly remove the PCR sample plate from Bravo deck position 4, leaving the red insert in place.



14 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

15 Quickly transfer the plate back to the thermal cycler, held at 65°C. On the thermal cycler, initiate the hybridization segment of the pre-programmed thermal cycler program (segment 4 from **Table 59** on page 96 or **Table 60** on page 96). During this step, the prepared DNA samples or DNA sample pools are hybridized to the Probe.

WARNING **The thermal cycler is held at 65°C using a heated lid at 105°C. The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.**

16 After initiating hybridization on the thermal cycler, click **Continue** on the VWorks screen.

17 To finish the VWorks protocol, click **Continue** in the Unused Tips and Empty Tip box dialogs, and click **Yes** in the Protocol Complete dialog.

18 When the Hybridization protocol is complete, set aside the Agilent Deep Well source plate containing the Blocker Mix and Fast Hybridization master mix located at position 6 of the Bravo deck for later use in the Post-CapPCR_SS_Max protocol. If you are using the pre-capture pooling workflow, remove the Eppendorf twin.tec plate containing the remainder of the prepared library pools. This plate is located at position 9 of the Bravo deck. Seal the plate and store it at –20°C in the event that the samples require further processing.

19 Continue to **Chapter 9**, "Capture and Amplification" on **page 119** for instructions on running the Capture & Wash protocol. Complete the setup tasks for that protocol (**step 1** on **page 120** through **step 3** on **page 122**) during the thermal cycler incubation for hybridization.

7 Hybridization (Fast)

Run the VWorks Hyb_SS_Max protocol

8 Hybridization (Overnight)

This chapter describes the steps to hybridize the DNA libraries or library pools to the Probe using the overnight hybridization method.

The NGS Workstation completes the liquid handling steps to set up the hybridization reactions using the Hyb_SS_Max automation protocol. First, the NGS Workstation combines the samples with the Blocker Mix. You then transfer the sample plate to the thermal cycler for sample denaturation and blocking prior to hybridization. Afterward, you transfer the sample plate back to the NGS Workstation for set up of the hybridization reactions. You then transfer the sample to the thermal cycler again to allow hybridization of the DNA samples to the probe. The hybridization segment of the thermal cycler program is 16 to 24 hours.

CAUTION The ratio of probe to prepped library is critical for successful capture.

This chapter uses the components listed in **Table 69**. Prepare each component as directed. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 69 Reagents thawed before use in Hyb_SS_Max protocol for overnight hybridization

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect Max Target Enrichment Kit Overnight Hyb Module Box 1, stored at RT	SureSelect Hyb 1 (bottle)	Keep at RT; vortex to mix	page 110
	SureSelect Hyb 2 (red cap)	Keep at RT; vortex to mix	page 110
	SureSelect Hyb 4 (black cap)	Keep at RT; vortex to mix	page 110
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 110
	SureSelect RNase Block (purple cap)	Thaw and keep on ice; vortex to mix	page 110
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 112
–80°C	SureSelect Probe	Thaw and keep on ice; vortex to mix	page 110

Pre-program the thermal cycler for overnight hybridization

1 Pre-program a thermal cycler with the appropriate overnight hybridization program.

- **Table 70** – hybridization program for the SureSelect XT Human All Exon V8 Probes (V8/V8+UTR/V8+NCV), SureSelect XT Human All Exon V7, and SureSelect XT PreCap Clinical Research Exome V4 Probe
- **Table 71** – hybridization program for all other probes

Set the heated lid to 105°C. Pre-warm the instrument before samples are loaded.

Table 70 Overnight hybridization program for the SureSelect XT Human All Exon V8/V8+UTR/V8+NCV, SureSelect XT Human All Exon V7, or SureSelect XT PreCap Clinical Research Exome V4 Probes (29 µL volume)

Segment	Purpose	Number of Cycles	Temperature	Time
1	Denaturation	1	95°C	5 minutes
2	Blocking	1	67.5°C	Hold* (at least 5 minutes)
3	Hybridization†	1	67.5°C	16–24 hours‡

* Samples are transferred to the NGS Workstation during this Hold step when prompted by the VWorks software.

† Keep samples at 67.5°C until they are processed in the Capture & Wash automation protocol that begins on **page 120**.

Table 71 Overnight hybridization program for *all other SureSelect XT probes* (29 µL volume)

Segment	Purpose	Number of Cycles	Temperature	Time
1	Denaturation	1	95°C	5 minutes
2	Blocking	1	65°C	Hold* (at least 5 minutes)
3	Hybridization†	1	65°C	16–24 hours‡

* Samples are transferred to the NGS Workstation during this Hold step when prompted by the VWorks software.

† Keep samples at 65°C until they are processed in the Capture & Wash automation protocol that begins on **page 120**.

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

Prepare the NGS Workstation for Hyb_SS_Max protocol

1 Clear the Labware MiniHub and BenchCel of all plates and tip boxes.

2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.

3 Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.

4 Place a red PCR plate insert at Bravo deck position 4.

Load the Labware MiniHub and BenchCel for Hyb_SS_Max protocol

NOTE

To avoid prolonged exposure of the master mix source plate to room temperature, load the Labware MiniHub and BenchCel Microplate Handling Workstation prior to preparation of the source plate.

1 Load the Labware MiniHub according to **Table 73**, using the plate orientations shown in **Figure 6** on page 59.

Table 72 Initial MiniHub configuration for Hyb_SS_Max protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	—	—	—	—
Shelf 1 (Bottom)	—	—	—	Empty tip box

2 Load the BenchCel Microplate Handling Workstation according to **Table 73**.

Table 73 Initial BenchCel configuration for Hyb_SS_Max protocol

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	2 Tip boxes	—	—	—
2	2 Tip boxes	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	2 Tip boxes	—	—	—
12	3 Tip boxes	—	—	—

Prepare the Hybridization Buffer

1 Prepare the appropriate volume of Hybridization Buffer by combining the reagents in **Table 74** at room temperature. Keep at room temperature until needed.

Table 74 Preparation of Hybridization Buffer

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Hyb 1 (bottle)	6.63 µL	97.3 µL	158.7 µL	220.2 µL	281.6 µL	404.6 µL	791.2 µL
SureSelect Hyb 2 (red cap)	0.27 µL	4.0 µL	6.5 µL	9.0 µL	11.5 µL	16.5 µL	32.2 µL
SureSelect Hyb 3 (yellow cap)	2.65 µL	38.9 µL	63.4 µL	88.0 µL	112.6 µL	161.7 µL	316.3 µL
SureSelect Hyb 4 (black cap)	3.45 µL	50.6 µL	82.6 µL	114.6 µL	146.6 µL	210.5 µL	411.7 µL
Total Volume	**13.0 µL**	**190.8 µL**	**311.2 µL**	**431.8 µL**	**552.3 µL**	**793.3 µL**	**1551.4 µL**

Prepare one or more Overnight Hybridization master mixes

1 Prepare the appropriate volume of Overnight Hybridization master mix for each of the Probes that will be used for hybridization as indicated in **Table 75** to **Table 78**. Mix by vortexing at medium speed for 15–20 seconds, then spin down briefly. Keep the Overnight Hybridization master mix(es) on ice.

NOTE

Each row of the prepped gDNA sample plate may be hybridized to a different Probe. However, Probes of different sizes require different post-capture amplification cycles. Plan experiments such that similar-sized Probes are hybridized on the same plate.

For runs that use a single Probe for all rows of the plate, prepare the master mix as described in Step a (**Table 75** or **Table 76**).

For runs that use different Probes for individual rows, prepare each master mix as described in Step b (**Table 77** or **Table 78**).

a For runs that use a single Probe for all rows, prepare a master mix as described in **Table 75** or **Table 76**, according to the probe design size.

b For runs that use different Probes in individual rows, prepare a master mix for each Probe as listed in **Table 77** or **Table 78**, according to the probe design size. The volumes listed in **Table 77** and **Table 78** are for a single row of sample wells. If a given Probe will be hybridized in multiple rows, multiply each of the values below by the number of rows assigned to that Probe.

Table 75 Preparation of Overnight Hybridization master mix for Probes <3 Mb, 8 rows of wells

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	62.3 µL	101.7 µL	141.1 µL	180.5 µL	259.3 µL	507.2 µL
SureSelect RNase Block (purple cap)	0.5 µL	6.9 µL	11.3 µL	15.7 µL	20.1 µL	28.8 µL	56.4 µL
Overnight Hybridization Buffer (prepared on **page 110**)	13 µL	180.1 µL	293.9 µL	407.7 µL	521.6 µL	749.2 µL	1465.2 µL
Probe (with design <3.0 Mb)	2 µL	27.7 µL	45.2 µL	62.7 µL	80.2 µL	115.3 µL	225.4 µL
Total Volume	**20 µL**	**277.0 µL**	**452.1 µL**	**627.2 µL**	**802.4 µL**	**1152.6 µL**	**2254.2 µL**

Table 76 Preparation of Overnight Hybridization master mix for Probes ≥3 Mb, 8 rows of wells

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	20.8 µL	33.9 µL	47.0 µL	60.2 µL	86.4 µL	169.1 µL
SureSelect RNase Block (purple cap)	0.5 µL	6.9 µL	11.3 µL	15.7 µL	20.1 µL	28.8 µL	56.4 µL
Overnight Hybridization Buffer (prepared on **page 110**)	13 µL	180.1 µL	293.9 µL	407.7 µL	521.6 µL	749.2 µL	1465.2 µL
Probe (with design ≥3.0 Mb)	5 µL	69.3 µL	113.1 µL	156.8 µL	200.6 µL	288.2 µL	563.6 µL
Total Volume	**20 µL**	**277.1 µL**	**452.2 µL**	**627.2 µL**	**802.5 µL**	**1152.6 µL**	**2254.2 µL**

Table 77 Preparation of Overnight Hybridization master mix for Probes <3 Mb, single row of wells

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	7.3 µL	12.0 µL	16.6 µL	21.2 µL	30.5 µL	59.7 µL
SureSelect RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.4 µL	3.4 µL	6.6 µL
Overnight Hybridization Buffer (prepared on **page 110**)	13 µL	21.2 µL	34.6 µL	48.0 µL	61.4 µL	88.1 µL	172.4 µL
Probe (with design <3 Mb)	2 µL	3.3 µL	5.3 µL	7.4 µL	9.4 µL	13.6 µL	26.5 µL
Total Volume	**20 µL**	**32.6 µL**	**53.2 µL**	**73.8 µL**	**94.4 µL**	**135.6 µL**	**265.2 µL**

Table 78 Preparation of Overnight Hybridization master mix for Probes ≥3 Mb, single row of wells

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	2.4 µL	4.0 µL	5.5 µL	7.1 µL	10.2 µL	19.9 µL
SureSelect RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.4 µL	3.4 µL	6.6 µL
Overnight Hybridization Buffer (prepared on page 110)	13 µL	21.2 µL	34.6 µL	48.0 µL	61.4 µL	88.1 µL	172.4 µL
Probe (with design ≥3 Mb)	5 µL	8.2 µL	13.3 µL	18.5 µL	23.6 µL	33.9 µL	66.3 µL
Total Volume	**20 µL**	**32.6 µL**	**53.2 µL**	**73.8 µL**	**94.5 µL**	**135.6 µL**	**265.2 µL**

Prepare the master mix source plate for Hyb_SS_Max protocol

1 Using an **Agilent Deep Well** plate, prepare the hybridization master mix source plate at room temperature, containing the Overnight Hybridization master mixes prepared in the previous step and the Blocker Mix. Add the volumes indicated in **Table 79** of each master mix to each well of the indicated column of the plate. When using multiple Probes in a run, add the Probe Hybridization master mix for each probe to the appropriate row(s) of the plate. The final configuration of the master mix source plate is shown in **Figure 15**.

Table 79 Preparation of the master mix source plate for Hyb_SS_Max protocol (overnight hybridization)

Master Mix Solution	**Position on Source Plate**	**Volume of master mix added per Well of Agilent Deep Well Source Plate**					
		1-Column Runs	**2-Column Runs**	**3-Column Runs**	**4-Column Runs**	**6-Column Runs**	**12-Column Runs**
Blocker Mix, ILM (blue cap)	Column 1 (A1-H1)	17.0 µL	22.5 µL	21.6 µL	28.8 µL	43.2 µL	83.5 µL
Overnight Hybridization master mix	Column 2 (A2-H2)	32.6 µL	53.2 µL	73.8 µL	94.4 µL	135.6 µL	265.2 µL

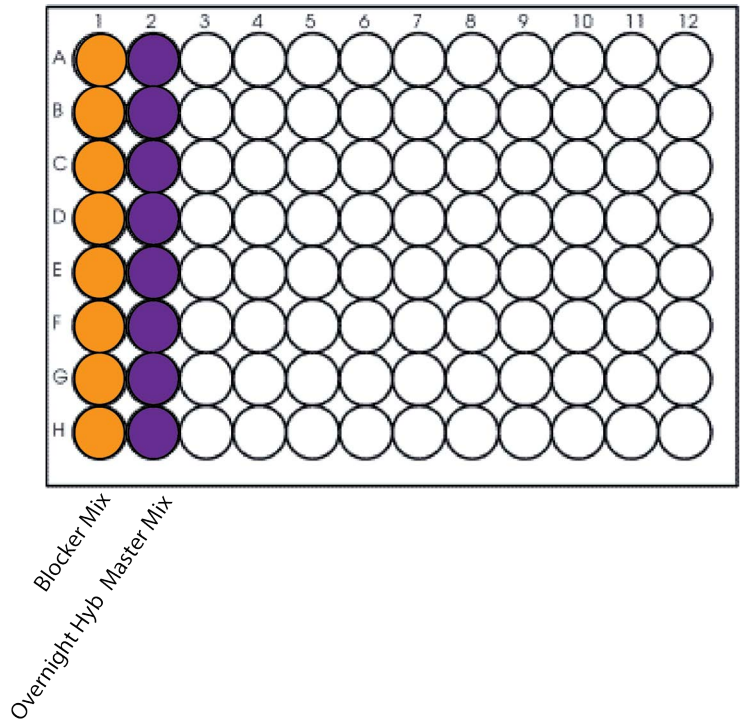


Figure 15 Configuration of the **Agilent Deep Well** master mix source plate for protocol Hyb_SS_Max. Column 2 can contain different Overnight Hybridization master mixes in each row.

2 Proceed immediately to loading the Bravo deck, keeping the master mix plate at room temperature only briefly during the loading process.

Load the Bravo deck for Hyb_SS_Max protocol

1 Load the Bravo deck according to **Table 80**.

Table 80 Initial Bravo deck configuration for Hyb_SS_Max protocol

Location	Content
4	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under step 3)
5	Empty Eppendorf twin.tec plate
6	Prepared library aliquots or library pools in Eppendorf twin.tec plate (unsealed)
8	Empty tip box
9	Agilent Deep Well Master Mix source plate (unsealed); seated in red insert

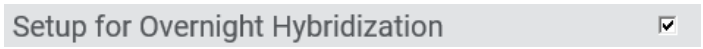
Run the VWorks Hyb_SS_Max protocol

1 On the SureSelect setup form, under **Select protocol to run**, select the **Hyb_SS_Max** protocol.

2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

3 Under **Select plate type for thermal cycling**, select the specific type of PCR plate used at position 4 of the Bravo deck.

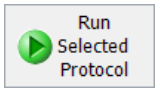
4 Mark the check box next to **Setup for Overnight Hybridization**.



5 Click **Display Initial Workstation Setup**.

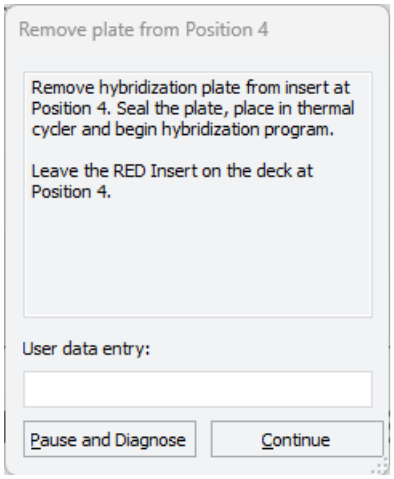
6 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

7 When verification is complete, click **Run Selected Protocol**.



The NGS Workstation combines the prepped gDNA in the wells of the sample plate with the aliquoted Blocker Mix. When this process is complete, you will be prompted to transfer the plate to the thermal cycler for sample denaturation and blocking prior to hybridization.

8 When prompted by VWorks as shown below, remove the PCR plate from position 4 of the Bravo deck, leaving the red insert in place. After removing the sample plate, click **Continue**.



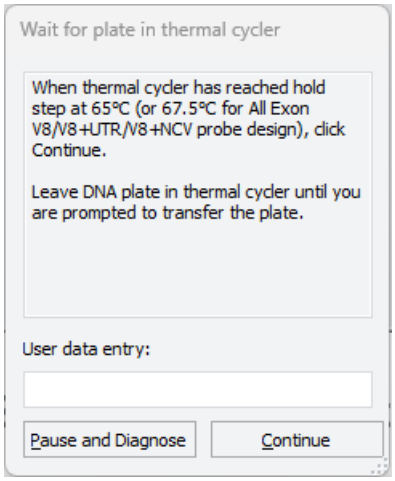
9 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

10 Transfer the sealed plate to a thermal cycler. Initiate the pre-programmed thermal cycler program (**Table 70** on page 108 or **Table 71** on page 108).

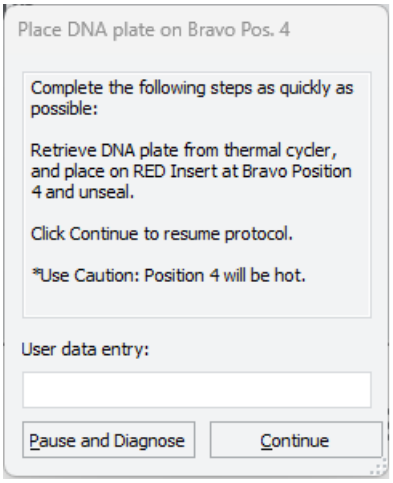
While the sample plate incubates on the thermal cycler, the NGS Workstation aliquots the Overnight Hybridization master mix to the Eppendorf twin.tec plate.

CAUTION You must complete **step 11** to **step 15** quickly, and immediately after being prompted by the VWorks software. It is important that sample temperature does not drop significantly during transfers between the NGS Workstation and thermal cycler.

11 When the NGS Workstation has finished aliquoting the Overnight Hybridization master mix, you will be prompted by VWorks as shown below. When the thermal cycler reaches Segment 2 (either 65°C or 67.5°C), click **Continue**. Leave the sample plate in the thermal cycler until you are notified to move it.



12 When prompted by VWorks as shown below, quickly remove the sample plate from the thermal cycler, unseal the plate carefully to avoid splashing, and transfer the plate to position 4 of the Bravo deck, seated in the red insert. Click **Continue**.



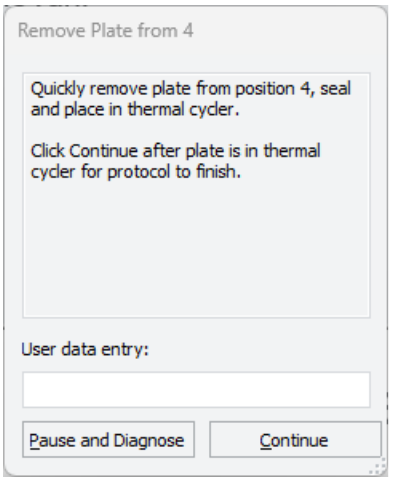
WARNING

Bravo deck position 4 will be hot.

Use caution when handling components that contact heated deck positions.

The NGS Workstation transfers the Overnight Hybridization master mix to the wells of the PCR plate that contain the mixture of prepped gDNA samples and blocking agents.

13 When prompted by VWorks as shown below, quickly remove the PCR sample plate from Bravo deck position 4, leaving the red insert in place.



14 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

15 Quickly transfer the plate back to the thermal cycler, held at 67.5°C or 65°C. On the thermal cycler, initiate the hybridization segment of the pre-programmed thermal cycler program (segment 3 from **Table 70** or **Table 71** on page 108). During this step, the prepared DNA samples or DNA sample pools are hybridized to the Probe.

WARNING **The thermal cycler is held at 65°C using a heated lid at 105°C. The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.**

16 After initiating hybridization on the thermal cycler, click **Continue** on the VWorks screen.

17 To finish the VWorks protocol, click **Continue** in the Unused Tips and Empty Tip box dialogs, and click **Yes** in the Protocol Complete dialog.

18 When the Hybridization protocol is complete, set aside the Agilent Deep Well source plate containing the Blocker Mix and Fast Hybridization master mix located at position 6 of the Bravo deck for later use in the Post-CapPCR_SS_Max protocol. If you are using the pre-capture pooling workflow, remove the Eppendorf twin.tec plate containing the remainder of the prepared library pools. This plate is located at position 9 of the Bravo deck. Seal the plate and store it at –20°C in the event that the samples require further processing.

19 After the thermal cycler program completes the next day, continue to **Chapter 9**, “Capture and Amplification” on **page 119** for instructions on running the Capture & Wash protocol.

8 Hybridization (Overnight)

Run the VWorks Hyb_SS_Max protocol

9 Capture and Amplification

This chapter describes the steps to capture and wash hybridized gDNA, amplify and purify the captured libraries, assess quality and quantity of the captured libraries, and pool libraries for multiplexed sequencing.

Step 1. Capture and wash the hybridized gDNA

This step uses runset Capture&Wash_SS_Max to automate capture of the gDNA-probe hybrids using streptavidin-coated magnetic beads.

NOTE For the fast hybridization workflow, setup tasks for the Capture & Wash protocol (**step 1**, below, through **step 3** on **page 122**) should be completed during the thermal cycler incubation for hybridization (approximately 1–2 hour duration) started on **page 104**.

The Capture & Wash runset uses the components listed in **Table 81**.

Table 81 Reagents for Capture and Washing

Storage Location	Kit Component	Preparative Steps	Where Used
+4°C	SureSelect Streptavidin Beads	Remove from 4°C just before use; vortex to mix	**page 121**
SureSelect Max Target Enrichment Kit Overnight Hyb Module Box 1, stored at RT -- OR -- SureSelect Max Target Enrichment Kit Fast Hyb Module Box 1, stored at RT	SureSelect Binding Buffer (bottle)	Ready to use	**page 121**
	SureSelect Wash Buffer 1 (bottle)	Ready to use	**page 121**
	SureSelect Wash Buffer 2 (bottle)	Ready to use	**page 121**

Prepare the NGS Workstation for Capture&Wash_SS_Max runset

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 75°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.

CAUTION Make sure to pre-set the temperature of Bravo deck position 4 prior to setting up the Capture & Wash runset. The NGS Workstation requires 20 to 25 minutes to reach 75°C.

4. Place a red PCR plate insert at Bravo deck position 4.

Prepare the Streptavidin beads

1 Vigorously resuspend the SureSelect Streptavidin Beads on a vortex mixer. The beads settle during storage.

2 Wash the magnetic beads.

a In a conical tube, combine the components listed in **Table 82**. The volumes below include the required overage.

Table 82 SureSelect Streptavidin Beads washing mixture

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Streptavidin Beads	40 µL	340 µL	660 µL	980 µL	1.32 mL	2.0 mL	4.0 mL
SureSelect Binding Buffer	160 µL	1.36 mL	2.64 mL	3.92 mL	5.28 mL	8.0 mL	16.0 mL
Total Volume	**200 µL**	**1.7 mL**	**3.3 mL**	**4.9 mL**	**6.6 mL**	**10 mL**	**20 mL**

b Mix the beads on a vortex mixer for 5 seconds.

c Put the conical tube into a magnetic separator device.

d Remove and discard the supernatant.

e Repeat **step a** through **step d** for a total of 3 washes. (Retain the beads after each wash and combine with a fresh aliquot of the indicated volume of SureSelect Binding Buffer.)

3 Resuspend the beads in SureSelect Binding Buffer, according to **Table 83** below.

Table 83 Preparation of magnetic beads for Capture&Wash_SS_Max runset

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Binding Buffer	160 µL	1.36 mL	2.64 mL	3.92 mL	5.28mL	8.0 mL	16.0 mL

4 Prepare an Eppendorf twin.tec source plate for the washed streptavidin bead suspension. For each well to be processed, add 160 µL of the homogeneous bead suspension to the Eppendorf twin.tec plate.

5 Place the streptavidin bead source plate at position 5 of the Bravo deck.

Prepare capture and wash solution source plates

1 Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water.

Make sure that the water in the reservoir does not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.

At the end of the automation protocol, retain this reservoir for use in the post-capture PCR bead cleanup protocol.

2 Prepare an Eppendorf twin.tec source plate labeled *Wash #1*. For each well to be processed, add 180 µL of SureSelect Wash Buffer 1.

3 Prepare an Agilent Deep Well source plate labeled *Wash #2*. For each well to be processed, add 1000 µL of SureSelect Wash Buffer 2.

Load the NGS Workstation for Capture&Wash_SS_Max runset

1 Load the Labware MiniHub according to **Table 84**, using the plate orientations shown in **Figure 6** on page 59.

Table 84 Initial MiniHub configuration for Capture&Wash_SS_Max runset

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	*Wash #1* Eppendorf twin.tec source plate	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	—	Nuclease-free water reservoir from **page 121**	—	—
Shelf 1 (Bottom)	—	—	—	Empty tip box

2 Load the Bravo deck according to **Table 85** (position 5 should already be loaded).

Table 85 Initial Bravo deck configuration for Capture&Wash_SS_Max runset

Location	Content
1	Empty waste reservoir (Agilent 2 mL square well)
4	Empty red insert
5	Streptavidin bead Eppendorf twin.tec source plate (added to deck on **page 121**)
6	*Wash #2* Deep Well source plate
8	Empty tip box

3 Load the BenchCel Microplate Handling Workstation according to **Table 86**.

Table 86 Initial BenchCel configuration for Capture&Wash_SS_Max runset

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	2 Tip boxes	—	—	—

Table 86 Initial BenchCel configuration for Capture&Wash_SS_Max runset

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
6	2 Tip boxes	—	—	—
12	4 Tip boxes	—	—	—

Run VWorks runset Capture&Wash_SS_Max

Start the Capture&Wash_SS_Max runset upon completion of the hybridization incubation.

The total duration of the Capture&Wash_SS_Max runset is approximately 2 hours. An operator must be present to complete two actions during the runset at the time points in the table below. The times provided are approximate; each action is completed in response to a VWorks prompt at the appropriate time in the runset.

Table 87 Operator actions during the Capture&Wash_SS_Max runset

Operator action	Approximate time after run start
Transfer hybridization reactions from thermal cycler to NGS Workstation	<5 minutes
Remove PCR plate from red aluminum insert	<5 minutes

4 On the SureSelect setup form, under **Select protocol to run**, select the **Capture&Wash_SS_Max** runset.

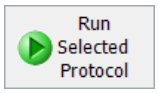
5 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

6 Under **Select plate type for thermal cycling**, select the specific type of PCR plate used for hybridization. This plate will be transferred from the thermal cycler to Bravo deck position 4 when prompted by VWorks.

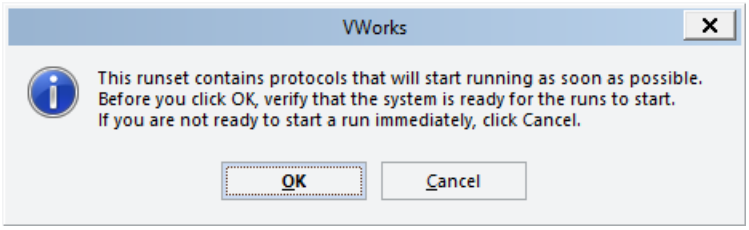
7 Click **Display Initial Workstation Setup**.

8 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

9 When verification is complete, click **Run Selected Protocol**.



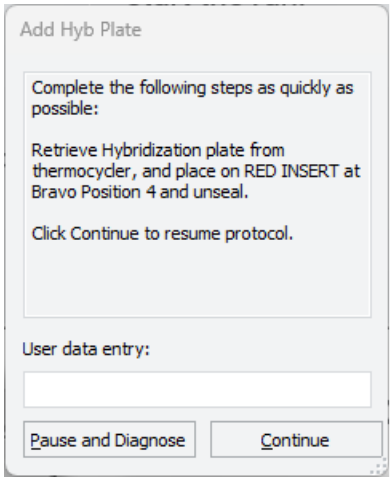
10 When ready to begin the run, click **OK** in the following window.



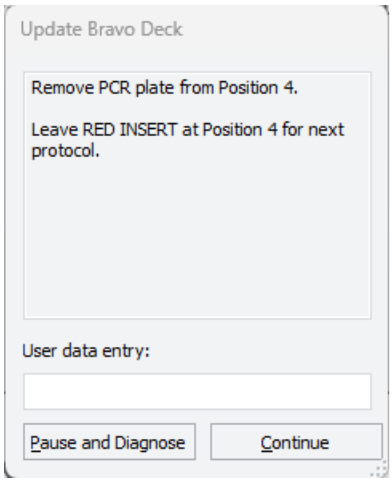
CAUTION

It is important to complete **step 11** quickly and carefully. Unseal the plate without tilting or jerking the plate to avoid sample splashing. Make sure that the NGS Workstation is completely prepared, with all components in place, before you transfer the sample plate to the Bravo deck.

11 When prompted by VWorks as shown below, quickly remove the PCR plate, containing the hybridization reactions, from the thermal cycler. Unseal the plate carefully to avoid splashing, and quickly transfer the plate to position 4 of the Bravo deck, seated in the red insert. Click **Continue** to resume the runset.



12 When prompted by VWorks as shown below, remove the PCR plate from position 4 of the Bravo deck, leaving the red aluminum insert in place. When finished, click **Continue** to resume the runset.



The remainder of the Capture&Wash_SS_Max runset takes approximately 2 hours. Once the runset is complete, the captured, bead-bound DNA samples are located in the Eppendorf plate at position 9 of the Bravo deck.

When the runset is complete, seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec and store the plate on ice while setting up the next automation protocol.

NOTE Captured DNA is retained on the streptavidin beads during the post-capture amplification step.

Step 2. Amplify the captured libraries

In this step, the NGS Workstation completes the liquid handling steps for PCR-amplification of the SureSelect-enriched DNA samples using the Post-CapPCR_SS_Max automated protocol. After the PCR plate is prepared by the NGS Workstation, you transfer the plate to a thermal cycler for amplification.

The design size of your Probe determines the amplification cycle number. Plan your experiments for amplification of samples prepared using probes of similar design sizes on the same plate. See **Table 90** on page 127 for cycle number recommendations.

This step uses the components listed in **Table 88**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 88 Reagents for post-capture PCR amplification

Storage Location	Kit Component	Preparative Steps	Where Used
SureSelect Max Target Enrichment Kit Fast Hyb Module Box 2, stored at –20°C -- OR -- SureSelect Max Target Enrichment Kit Overnight Hyb Module Box 2, stored at –20°C	Amplification Master Mix (bottle)	Thaw on ice then keep on ice. Mix thoroughly by inversion at least 5X. Do not vortex.	**page 127**
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 127**

CAUTION To avoid cross-contaminating libraries, set up PCR master mixes in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

Prepare the NGS Workstation for Post-CapPCR_SS_Max protocol

1. Clear the Labware MiniHub and BenchCel of plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
5. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
6. Place a red PCR plate insert at Bravo deck position 6.

Pre-program the thermal cycler for post-capture PCR

1 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 89**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

Table 89 Post-capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	45 seconds
2	10 to 16 See **Table 90** for recommendations based on probe design size	98°C	15 seconds
		60°C	30 seconds
		72°C	30 seconds
3	1	72°C	1 minute
4	1	4°C	Hold

Table 90 Post-capture PCR cycle number recommendations

Probe Size/Description	Cycles
Probes <0.2 Mb	16 cycles
Probes 0.2–3 Mb	12–16 cycles
Probes 3–5 Mb	11–12 cycles
Probes >5 Mb (including Human All Exon and Exome Probes)	10–11 cycles

Prepare the post-capture PCR master mix and master mix source plate

1 Prepare the appropriate volume of post-capture PCR Master Mix, according to **Table 91**. Mix well using a vortex mixer and keep on ice.

Table 91 Preparation of Post-Capture PCR Master Mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Amplification Master Mix (bottle)	25 µL	343.3 µL	563.9 µL	709.9 µL	946.3 µL	1419.4 µL	2770.7 µL
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	13.7 µL	22.6 µL	28.4 µL	37.9 µL	56.8 µL	110.8 µL
Total Volume	**26 µL**	**357.0 µL**	**586.5 µL**	**738.3 µL**	**984.2 µL**	**1476.2 µL**	**2881.5 µL**

2 Using an Agilent Deep Well master mix source plate, prepare the master mix source plate by adding the volume of PCR master mix indicated in **Table 92** to all wells of column 3 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 16**

You can use the same Agilent Deep Well plate that was used as the master mix source plate in the Hyb_SS_Max protocol if that protocol was run on the same day.

Table 92 Preparation of the master mix source plate for Post-CapPCR_SS_Max protocol

Master Mix Solution	Position on Source Plate	Volume of master mix added per well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Post-Capture PCR Master Mix	Column 3 (A3-H3)	42.0 µL	69.0 µL	91.2 µL	121.5 µL	177.0 µL	339.0 µL

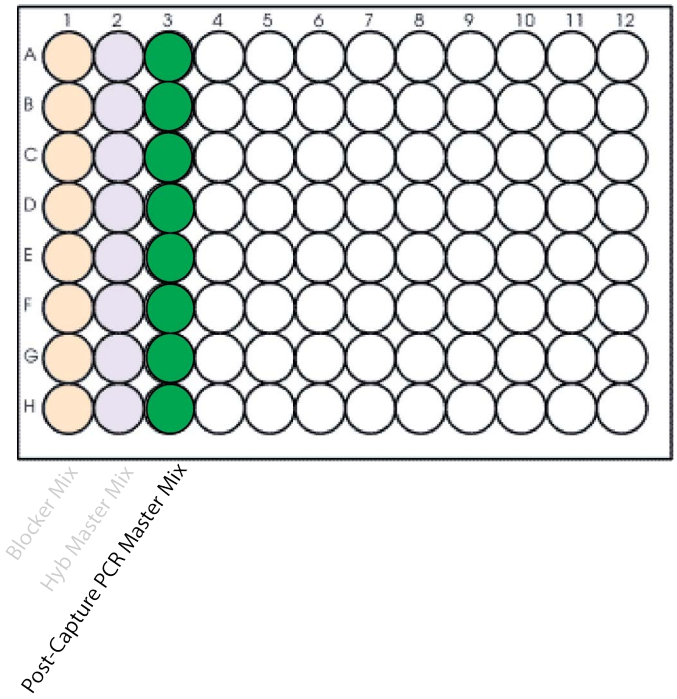


Figure 16 Configuration of the **Agilent Deep Well** master mix source plate for protocol Post-Cap-PCR_SS_Max. The mixes dispensed into columns 1 and 2 during previous protocols are shown in light shading.

3 Seal the master mix source plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

4 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

Load the NGS Workstation for Post-CapPCR_SS_Max protocol

1 Load the Labware MiniHub according to **Table 93**, using the plate orientations shown in **Figure 6** on page 59.

Table 93 Initial MiniHub configuration for Post-CapPCR_SS_Max protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	New tip box	—	—	—
Shelf 1 (Bottom)	Empty tip box	—	—	Empty tip box

2 Load the Bravo deck according to **Table 94**.

Table 94 Initial Bravo deck configuration for Post-CapPCR_SS_Max protocol

Location	Content
4	Captured DNA bead suspensions in Eppendorf twin.tec plate (unsealed)
6	Empty PCR plate; seated in red insert (PCR plate type must be specified on setup form under step 3)
9	Deep Well master mix plate containing PCR Master Mix in Column 3 (unsealed)

3 Load the BenchCel Microplate Handling Workstation according to **Table 95**.

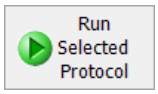
Table 95 Initial BenchCel configuration for Post-CapPCR_SS_Max protocol

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Run VWorks protocol Post-CapPCR_SS_Max

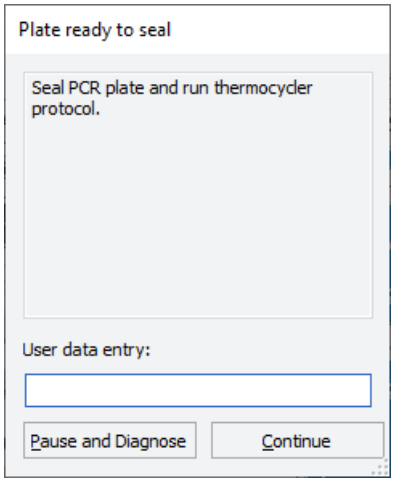
1. On the SureSelect setup form, under **Select protocol to run**, select the **Post-CapPCR_SS_Max** protocol.
2. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
3. Under **Select plate type for thermal cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.
4. Click **Display Initial Workstation Setup**.

5. Verify that the NGS Workstation has been set up as displayed at the bottom of the form.
6. When verification is complete, click **Run Selected Protocol**.



Running the Post-CapPCR_SS_Max protocol takes approximately 15 minutes. Once complete, the PCR-ready samples, containing captured DNA and PCR master mix, are located in the PCR plate at position 6 of the Bravo deck.

7. When you see the following prompt, remove the PCR plate from position 6 of the Bravo deck, then click **Continue**. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 seconds.



8. Place the plate in the thermal cycler. Resume the thermal cycler program in **Table 89** on page 127.
9. When the PCR amplification program is complete, spin the plate briefly then keep on ice until it is loaded on the Bravo deck for the next protocol..

Step 3. Purify the amplified indexed libraries using SureSelect Max Purification Beads

In this step, the NGS Workstation transfers SureSelect Max Purification Beads to the indexed DNA sample plate and then collects and washes the bead-bound DNA using the Bead_Cleanup_SS_Max (Post-Capture PCR) automated protocol.

This step uses the aliquoted plate of SureSelect Max Purification Beads that was prepared on **page 52**.

Prepare the NGS Workstation for Bead_Cleanup_SS_Max (Post-Capture PCR) protocol

1. Set aside the Agilent Deep Well source plate containing the Post-Capture PCR master mix located at position 9 of the Bravo deck for later use in the TS_HighSensitivity_D1000 protocol (see **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and High Sensitivity D1000 ScreenTape"** on page 134). Otherwise, clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.

Prepare the purification reagents for Bead_Cleanup_SS_Max (Post-Cap PCR) protocol

1. Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water. Use the same Agilent shallow well reservoir that was used in the Capture&Wash_SS_Max protocol.

   Make sure that the water in the reservoir does not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.
2. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.

Load the NGS Workstation for Bead_Cleanup_SS_Max (Post-Capture PCR) protocol

1 Load the Labware MiniHub according to **Table 96**, using the plate orientations shown in **Figure 6** on page 59.

Table 96 Initial MiniHub configuration for Bead_Cleanup_SS_Max (Post-Capture PCR) protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Aliquoted purification beads in Eppendorf twin.tec plate from **page 52** (50 µL of beads/well)	—	—	—
Shelf 4	Empty Eppendorf twin.tec Plate	—	—	—
Shelf 3	—	—	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 1**		—
Shelf 1 (Bottom)	—	70% ethanol reservoir from **step 2**	—	Empty tip box

2 Load the Bravo deck according to **Table 97**.

Table 97 Initial Bravo deck configuration for Bead_Cleanup_SS_Max (Post-Capture PCR) protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	Amplified DNA libraries or library pools in unsealed PCR plate; seated in red insert (PCR plate type must be specified on setup form under step 3)

3 Load the BenchCel Microplate Handling Workstation according to **Table 98**.

Table 98 Initial BenchCel configuration for Bead_Cleanup_SS_Max (Post-Capture PCR) protocol

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	3 Tip boxes	—	—	—
12	5 Tip boxes	—	—	—

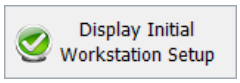
Run VWorks protocol Bead_Cleanup_SS_Max (Post-Capture PCR)

1 On the SureSelect setup form, under **Select protocol to run**, select the **Bead_Cleanup_SS_Max (Post-Capture PCR)** protocol.

2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

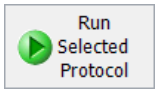
3 Under **Select plate type for thermal cycling**, select the specific type of PCR plate containing the amplified libraries at position 9.

4 Click **Display Initial Workstation Setup**.



5 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

6 When verification is complete, click **Run Selected Protocol**.



The purification protocol takes approximately 45 minutes. When complete, the amplified DNA samples are in the Eppendorf plate located on Bravo deck position 7.

Step 4. Assess sequencing library DNA quantity and quality

Post-capture library analysis can be done using one of two options.

- Option 1: Prepare the analytical assay plate using automation (protocol TS_HighSensitivity_D1000) and perform analysis on Agilent 4200 TapeStation. See **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and High Sensitivity D1000 ScreenTape"** on page 134.
- Option 2: Prepare the analytical samples manually and perform analysis on Agilent 4200 or 4150 TapeStation or Agilent 5200 Fragment Analyzer. See **"Option 2: Analysis using an equivalent platform (non-automated)"** on page 138.

Option 1: Analysis using an Agilent 4200 TapeStation Instrument and High Sensitivity D1000 ScreenTape

This section describes the use of automation protocol TS_HighSensitivity_D1000 to prepare samples for analysis. The automation protocol prepares the assay sample plate by combining 3 µL of each DNA sample with 3 µL of High Sensitivity D1000 Sample Buffer. Afterward, you transfer the sample plate to the 4200 TapeStation instrument for analysis. For more information to do this step, see the Agilent High Sensitivity D1000 Assay Quick Guide for 4200 TapeStation System.

Allow the reagents used with the analysis system to equilibrate to room temperature for 30 minutes prior to use.

Prepare the NGS Workstation and Sample Buffer source plate

1. Clear the Labware MiniHub and BenchCel of plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.
3. Turn off the ThermoCube device (see **page 24**) to restore position 9 of the Bravo deck to room temperature.
4. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
5. Using the same **Agilent Deep Well** master mix source plate that was used for protocol Post-CapPCR_SS_Max, prepare the Sample Buffer source plate at room temperature. Add the volume of High Sensitivity D1000 Sample Buffer indicated in **Table 99** to each well of column 4 of the plate. The final configuration of the Sample Buffer source plate is shown in **Figure 17**.

Table 99 Preparation of the Sample Buffer Source Plate for TS_HighSensitivity_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
High Sensitivity D1000 Sample Buffer	Column 4 (A4-H4)	8 µL	11 µL	14 µL	17 µL	24 µL	44 µL

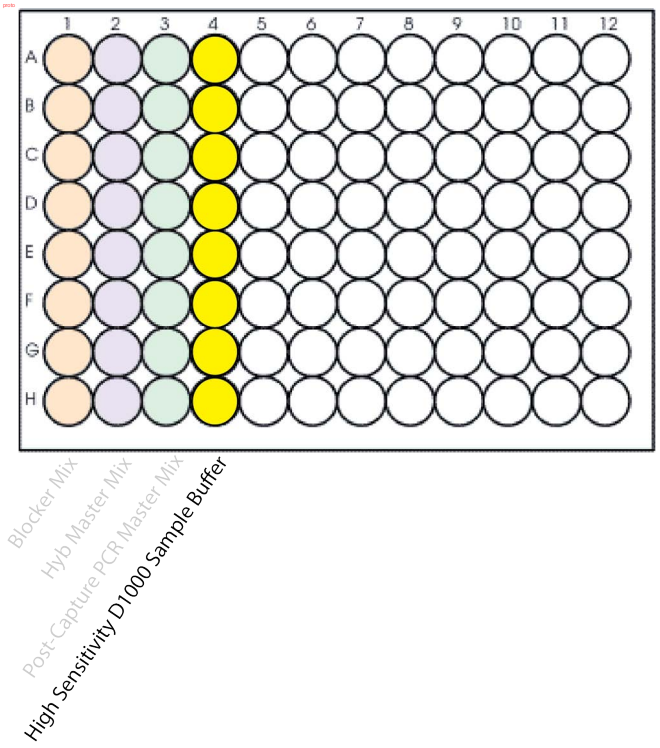


Figure 17 Configuration of the **Agilent Deep Well** source plate for protocol TS_High-Sensitivity_D1000. The mixes dispensed into columns 1–3 during previous protocols are shown in light shading.

Load the NGS Workstation for TS_HighSensitivity_D1000 protocol

6 Load the Labware MiniHub according to **Table 100**, using the plate orientations shown in **Figure 6** on page 59.

Table 100 Initial MiniHub configuration for TS_HighSensitivity_D1000 protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	New tip box	—	—	—
Shelf 1 (Bottom)	Empty tip box	—	—	Empty tip box

7 Load the Bravo deck according to **Table 101**.

Table 101 Initial Bravo deck configuration for TS_HighSensitivity_D1000 protocol

Location	Content
4	Amplified post-capture libraries or library pools in Eppendorf twin.tec plate (unsealed)
6	Empty Eppendorf twin.tec plate
9	Agilent Deep Well source plate containing High Sensitivity D1000 Sample Buffer in Column 4

CAUTION

To prevent damage to the Agilent 4200 TapeStation instrument and the NGS Workstation, use only the specified Eppendorf twin.tec plates (Eppendorf p/n 951020401 or 951020619) for automated assay plate preparation.

The Agilent 2200 TapeStation system does not support use of the Eppendorf twin.tec plates. Do not load sample plates prepared using the automated protocol in the 2200 TapeStation instrument.

8 Load the BenchCel Microplate Handling Workstation according to **Table 102**.

Table 102 Initial BenchCel configuration for TS_HighSensitivity_D1000 protocol

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

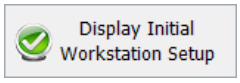
Run VWorks protocol TS_HighSensitivity_D1000

9 In the VWorks software, open the Utility form. See **"Accessing the Supplemental VWorks Forms"** on page 30.

10 Under **Select protocol to run**, select the **TS_HighSensitivity_D1000** protocol.

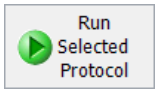
11 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

12 Click **Display Initial Workstation Setup**.



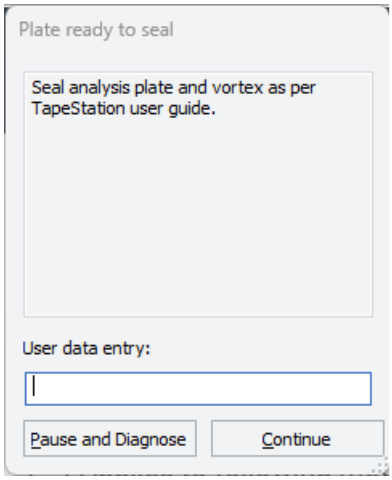
13 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

14 When verification is complete, click **Run Selected Protocol**.



Running the TS_HighSensitivity_D1000 protocol takes approximately 15 minutes. Once removal of analytical samples is complete, remove the primary DNA library sample plate from position 4, seal the plate, and keep on ice.

15 When prompted by VWorks as shown below, remove the Eppendorf twin.tec plate containing the analytical samples from position 6 of the Bravo deck, then click **Continue**. Seal the assay plate with a foil seal and vortex the sealed plate as directed in the Agilent High Sensitivity D1000 Assay Quick Guide for 4200 TapeStation System.



CAUTION

To prevent damage to the Agilent 4200 TapeStation instrument, use only the specified 96-well plate foil seals (Agilent p/n 5067-5154).

For accurate quantitation, make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 1 minute, then spin briefly to collect the liquid.

Run the High Sensitivity D1000 Assay and analyze the data

16 Load the analytical sample plate, the High Sensitivity D1000 ScreenTape, and loading tips into the TapeStation as instructed in the instrument user manual. Start the run.

17 Using the 150 bp to 1000 bp region of the electropherogram, determine the average fragment size and the library concentration (see **Table 103** for guidelines). Sample electropherograms are shown in **Figure 18** (library prepared from high-quality DNA using overnight hybridization) and **Figure 19** (library prepared from FFPE-derived DNA using overnight hybridization).

Table 103 Post-capture library qualification guidelines

Input type	NGS read length used to select fragmentation duration	Expected average fragment size (150–1000 bp region)
Intact DNA	2 × 100 reads	350 to 450 bp
	2 × 150 reads	380 to 480 bp
FFPE DNA	2 × 100 reads OR 2 × 150 reads	250 to 390 bp

18 Determine the concentration of each library by integrating under the entire peak.

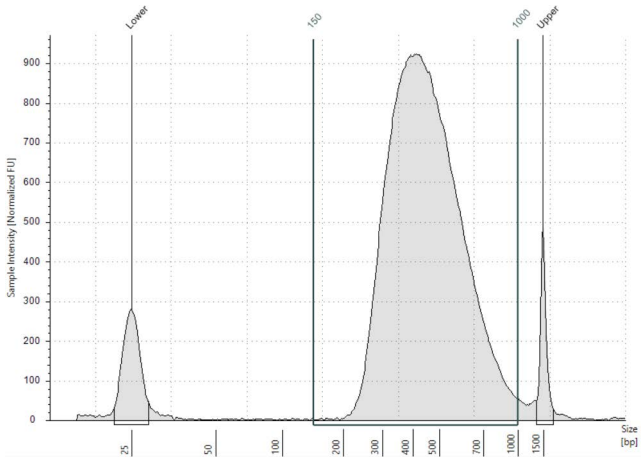


Figure 18 Post-capture library prepared from a high-quality gDNA sample using overnight hybridization, analyzed in a HighSensitivity D1000 ScreenTape assay.

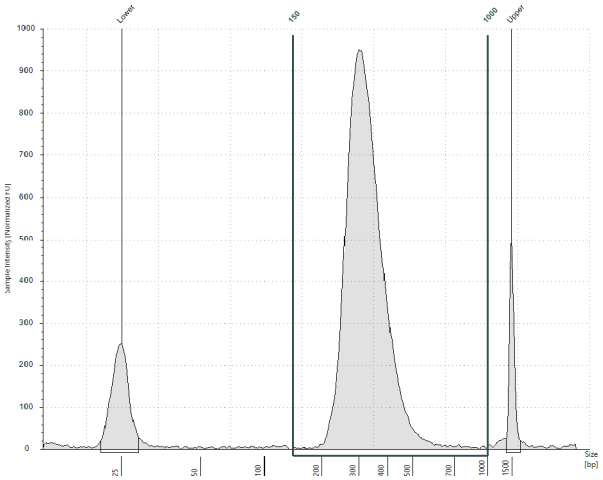


Figure 19 Post-capture library prepared from an FFPE-derived DNA sample using overnight hybridization, analyzed in a High Sensitivity D1000 ScreenTape assay.

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

Option 2: Analysis using an equivalent platform (non-automated)

Using manual preparation of the analytical samples, you can analyze the DNA samples on an Agilent 5200 Fragment Analyzer or Agilent 4200/4150 TapeStation. Electropherograms obtained using one of these other analysis platforms are expected to show fragment size profiles similar to those shown for the Agilent 4200 TapeStation (see **Figure 18** through **Figure 19**). Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 103** for guidelines). **Table 104** includes links to assay instructions.

Table 104 Post-capture library analysis options

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

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 5. Optional: Pool samples for multiplexed sequencing

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 Primers 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 libraries such that each index-tagged sample is present in equimolar amounts in the pool using one of the two methods described below. **Method 2** can use the Aliquot_Captures automation protocol to pool samples. Use the diluent specified by your sequencing provider, such as Low TE, for the dilution steps

Method 1 Dilute each indexed library to be pooled to the same final concentration (typically 4 nM to 15 nM, or the concentration of the most dilute sample). This dilution step is performed by manually pipetting the diluent directly into the wells of the source plate. Then, combine equal volumes of all libraries to create the final pool in the destination plate.

Method 2 Starting with indexed libraries at different concentrations, add the appropriate volume of each library to the destination well (either manually or using the Aliquot_Captures automation protocol) to achieve equimolar concentration in the pool. Then, adjust the pool to the desired final volume by adding the appropriate volume of diluent to each well. This volume adjustment is performed by manually pipetting the diluent directly into the wells of the destination plate. The formula below is provided for determination of the amount of each indexed sample to add to the pool.

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

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

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

is the number of indexes, and

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

Table 105 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.

Table 105 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

Pool samples for multiplexed sequencing using automation (optional for Method 2)

The instructions below are for **Method 2**. Alternatively, you can perform this method using an entirely manual approach rather than with the Aliquot_Captures automation protocol.

1. Create a CSV (comma separated value) file with the headers shown in **Figure 20**. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in CSV format. The file must include rows for all 96 wells of the plate.
2. Enter the information requested in the header for each DNA sample. **Figure 20** shows an example spreadsheet.
   - In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
   - In the SourceWell field, enter each well position on the source plate containing an amplified and captured indexed library that needs to be added to a pool. Use the Eppendorf twin.tec plate containing the purified indexed libraries as the source plate.
   - In the DestinationWell field, enter the well position on the destination plate for the pool.
   - In the Volume field, enter the volume (in µL) of each indexed library to be transferred from the source well to the destination well. The volume for each library is calculated from its concentration.
   - For all empty wells on the source plate, delete the corresponding rows in the CSV file.

	A	B	C	D
1	SourceBC	SourceWell	DestinationWell	Volume
2	abc	A1	A1	4.711292
3	abc	B1	A1	6.37105
4	abc	C1	A1	7.000448
5	abc	D1	A1	3.81144
6	abc	E1	A1	9.539072
7	abc	F1	A1	7.802747
8	abc	G1	A1	8.835171
9	abc	H1	A1	6.313131
10	abc	A2	A1	5.976286
11	abc	B2	A1	6.601183
12	abc	C2	A1	7.14449
13	abc	D2	A1	5.66431

Figure 20 Sample spreadsheets for method 1 and method 2

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\Agilent_SS_Max_DNA_B1.1.1.2\Aliquot Input File Templates\Aliquot_Captures_Template.csv**.

The Aliquot_Captures_Template.csv file may be copied and used as a template for creating the CSV files for each Aliquot_Captures protocol run. If you are using the sample file as a template for runs with fewer than 12 columns, be sure to delete rows for any unused columns on the plate.

3 Load the CSV file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B\Agilent_SS_Max_DNA_B1.1.1.2\Aliquot Input File Templates**.

4 Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.

5 Load the Bravo deck according to **Table 106**.

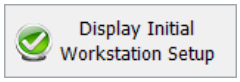
Table 106 Initial Bravo deck configuration for Aliquot_Captures protocol

Location	Content
5	Empty Agilent Deep Well plate
6	Empty tip box
8	New tip box
9	Purified amplified indexed libraries in Eppendorf twin.tec plate

6 In the VWorks software, open the Utility form. See **"Accessing the Supplemental VWorks Forms"** on page 30.

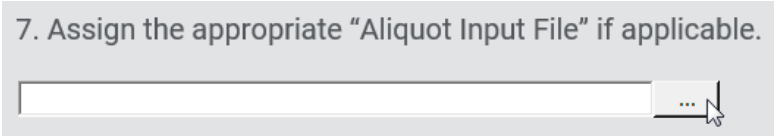
7 Under **Select protocol to run** select the **Aliquot_Captures** protocol.

8 Click **Display Initial Workstation Setup**.



9 Upload the CSV file created in **step 1** through **step 3** (page **141** to **142**).

a Click the "**...**" browse button below **Assign the appropriate "Aliquot Input File" if applicable** to open a directory browser window.

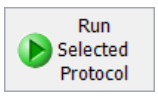


b Browse to the location where you saved the CSV file. Select the file and click **Open**.

The directory browser window closes, returning you to the SureSelect setup form. The selected file location is listed in the field.

10 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

11 When verification is complete, click **Run Selected Protocol**.



The aliquoting protocol takes approximately 1 hour for 96 samples. When complete, the destination plate containing the library pools is on Bravo deck position 5.

12 Remove the destination plate from the Bravo deck.

13 Add the appropriate volume of diluent to each well to bring the pool to the necessary DNA concentration for sequencing.

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

10 NGS and Analysis Guidelines

This chapter 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 21**. 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.

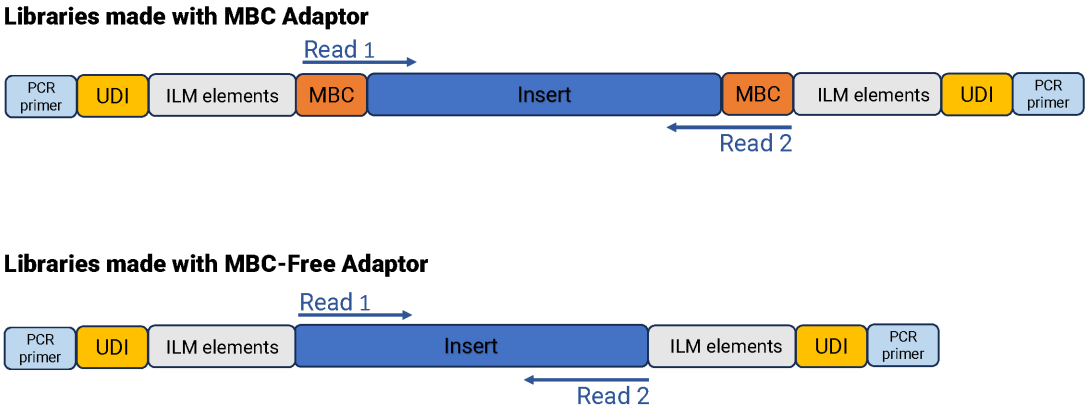


Figure 21 Content of SureSelect Max for ILM sequencing libraries. Each fragment contains one target insert (dark blue) surrounded by the following elements: Illumina paired-end sequencing elements (gray), unique dual indexes (gold), library PCR primers (light blue) and optional molecular barcode (MBC) 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 107** 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 107 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 107** 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 108** showing example settings for 2x150 bp sequencing.

Table 108 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 150**.
- 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 libraries. Your NGS pipeline may vary.

MBC-free libraries

1 **Demultiplexing**: Generate demultiplexed FASTQ files for each sample using Illumina's bcl2fastq, BCL Convert, or a similar software application. This process generates paired-end reads based on the dual indexes and removes sequences with incorrectly paired P5 and P7 indexes.

2 **Adaptor trimming**: Turn on the adaptor trimming tools in the selected Illumina demultiplexing software to complete adaptor trimming at this step.

3 **Alignment**: The trimmed reads should be aligned using a suitable tool such as BWA-MEM.

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

MBC-tagged libraries

1 **Demultiplexing**: Generate demultiplexed FASTQ files for each sample using Illumina's bcl2fastq, BCL Convert, or a similar software application. This process generates paired-end reads based on the dual indexes and removes sequences with incorrectly paired P5 and P7 indexes.

   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.

NOTE

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.

If demultiplexing using bcl2fastq, MBCs may be masked by including the base mask **N5Y*,I8,I8,N5Y*** (where * is replaced with the remaining read length after subtracting the 5 masked bases, e.g., use **N5Y146,I8,I8,N5Y146** for 2x150 NGS set up as shown in **Table 108** on page 147). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

If demultiplexing using BCL Convert, MBCs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y*;I8;I8;N5Y*** (where * is replaced with read length after trimming, e.g., use **N5Y146;I8;I8;N5Y146** for 2x150 NGS set up as shown in **Table 108** on page 147). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

Alternatively, the first 5 bases may be trimmed from the demultiplexed FASTQ files using a suitable processing tool (e.g., fgbio).

2 **MBC-adaptor processing**: Use a suitable processing tool of your choice to trim and collect inline MBCs from each sequencing read. For example, MBC processing could be conducted with the fgbio best practice consensus pipeline, as described in: https://github.com/fulcrumgenomics/fgbio/blob/main/docs/best-practice-consensus-pipeline.md. The output includes trimmed, deduplicated reads in unaligned BAM format with single-stranded MBC consensus reads. For generation of double-stranded MBC consensus reads, refer to https://fulcrumgenomics.github.io/fgbio/tools/latest/CallDuplexConsensusReads.html.

   - Inline MBCs are added to both ends of the DNA inserts in the assay. To collect the MBCs and process them for analysis, one approach is to trim off 5 bases at the beginning of each read, take the first 3 of the 5 bases as MBC, and discard the remaining 2.

3 **Alignment**:

   - DNA library alignment can be completed as part of the fgbio best practice consensus pipeline described at the link above or aligned using another suitable alignment tool of your choice.

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

SureSelect Max UDI Information

The SureSelect Max unique dual indexes (UDIs) are added to the library fragments during library preparation. Each fragment contains a unique 8-bp P5 and P7 index (see **Figure 21** on page 146) 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 151** through **page 158**. 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 110** through **Table 117** 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 109**. 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 109 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 110 SureSelect Max Index Primer Pairs 1–48, 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
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 111 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 112 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 113 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 114 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 115 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 116 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 117 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

11 Appendix

This appendix provides instructions for using the Aliquot_Libraries automation protocol to prepare a sample plate for single-plex hybridization.

Using the Aliquot_Libraries automated protocol

For the post-capture pooling workflow, the Eppendorf plate prepared during the Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol is used as the sample plate in the Hyb_SS_Max protocol for single-plex hybridization. The NGS Workstation removes an aliquot of each DNA sample from the sample plate for use in the Hyb_SS_Max protocol. The quantity of DNA in the aliquot varies from sample to sample.

However, if you are using fast hybridization, you have the option to use a specific quantity of DNA in the hybridization reactions. Use the Aliquot_Libraries automation protocol on the Utility form in VWorks to prepare a new Eppendorf sample plate containing the desired quantity of DNA (minimum of 500 ng) in the required 12-µL volume.

Prepare the CSV file

Before running the Aliquot_Libraries automation protocol, you must create a table containing instructions for the NGS Workstation indicating the volume of each sample to aliquot, as described in the steps below.

1. Create a CSV (comma separated value) file with the headers shown in **Figure 22**. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in CSV format. The file must include rows for all 96 wells of the plate.
2. Enter the information requested in the header for each DNA sample.
   - In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
   - In the SourceWell and DestinationWell fields, enter each well position for the plate. SourceWell and DestinationWell field contents must be identical for a given sample.
   - In the Volume field, enter the volume (in µL) of each DNA sample to be used in the hybridization step. For all empty wells on the plate, delete the corresponding rows in the CSV file. Maximum volume is 12 µL.

	A	B	C	D
1	SourceBC	SourceWell	DestinationWell	Volume
2	abc	A1	A1	5.35
3	abc	B1	B1	4.28
4	abc	C1	C1	5.19
5	abc	D1	D1	4.76
6	abc	E1	E1	5.19
7	abc	F1	F1	5.49
8	abc	G1	G1	4.86
9	abc	H1	H1	5.05
10	abc	A2	A2	4.37

Figure 22 Sample spreadsheet for 1-column run

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\Agilent_SS_Max_DNA\Aliquot Input File Templates\Aliquot_Libraries_Template.csv**.

The Aliquot_Libraries_template.csv file may be copied and used as a template for creating the CSV file for each Aliquot_Libraries protocol run. If you are using the sample file as a template for runs with fewer than 12 columns, be sure to delete rows for any unused columns on the plate.

3 Load the CSV file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B\Agilent_SS_Max_DNA\Aliquot Input File Templates**.

Prepare and load the NGS Workstation

1 Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.

2 Load the Bravo deck according to **Table 118**.

Table 118 Initial Bravo deck configuration for Aliquot_Libraries protocol

Location	Content
2	Nuclease-free water in Agilent shallow well reservoir (30 mL)
5	Empty Eppendorf twin.tec plate
6	Empty tip box
8	New tip box
9	Prepped library DNA in Eppendorf twin.tec plate prepared during the Bead_Cleanup_SS_Max (Pre-Cap PCR) protocol

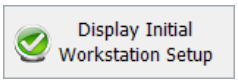
Run the VWorks Aliquot_Libraries protocol

1 In the VWorks software, open the Utility form. See **"Accessing the Supplemental VWorks Forms"** on page 30.

2 Under **Select protocol to run**, select the **Aliquot_Libraries** protocol.

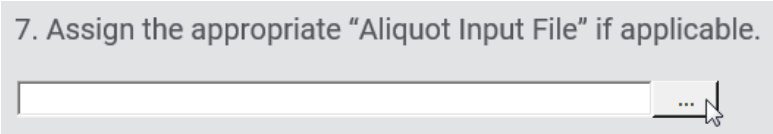
3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

4 Click **Display Initial Workstation Setup**.



5 Upload the CSV file created on **page 160**.

   a Click the "**...**" browse button below **Assign the appropriate "Aliquot Input File" if applicable** to open a directory browser window.

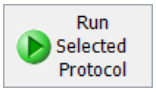


   b Browse to the location where you saved the CSV file. Select the file and click **Open**.

   The directory browser window closes, returning you to the Utility form. The selected file location is listed in the field.

6 Verify that the NGS Workstation has been set up as displayed at the bottom of the form.

7 When verification is complete, click **Run Selected Protocol**.



The library aliquoting protocol takes approximately 1 hour for 96 samples. When complete, the DNA sample plate is on Bravo deck position 5.

Remove the sample plate from the Bravo deck. Proceed directly to **Chapter 7**, "Hybridization (Fast)," starting on page 95.

12 Reference

This chapter contains reference information, including component kit contents, index sequences, and troubleshooting information.

Reagent Kit Contents

Library Preparation

SureSelect Max DNA Library Preparation with enzymatic fragmentation uses the kits listed in **Table 119**. Contents of the multi-part component kits are shown in **Table 120** through **Table 122**.

Table 119 Kits for SureSelect Max DNA Library Preparation with Enzymatic Fragmentation

Purchased Kit	Included Component Kits	Component Kit Part Number for 96-reaction kit	Storage Condition
SureSelect Max Enzymatic Fragmentation Library Preparation Kit	SureSelect Max Enzymatic Fragmentation Library Preparation Module	5280-0064	–20°C
SureSelect Max Adaptors and UDI Primers Kit for ILM	SureSelect Max MBC Adaptor Oligo Mix for ILM **OR** SureSelect Max MBC-Free Adaptor Oligo Mix for ILM	5282-0125 **OR** 5282-0127	–20°C
	SureSelect Max UDI Primers for ILM	5282-0120 (Index 1-96) 5282-0121 (Index 97-192) 5282-0122 (Index 193-288) 5282-0123 (Index 289-384)	–20°C
SureSelect Max Purification Beads		5282-0226	+4°C

Component Kit Details

Table 120 SureSelect Max Library Preparation Module content

Kit Component	96 Reaction Kit (p/n 5280-0064)
Frag/A-Tail Enzyme Mix	tube with green cap
Frag/A-Tail Buffer	tube with yellow cap
Ligation Master Mix	bottle
Amplification Master Mix	bottle

Table 121 SureSelect Max Adaptor Oligo Mix for ILM options

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

Table 122 SureSelect Max UDI Primers for ILM options

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

* See page 166 for index plate position maps; see page 151 for index pair sequences.

Target Enrichment

Agilent offers SureSelect Max Target Enrichment kits for either fast or overnight hybridization. Both kits are listed in **Table 123**. Detailed contents of each of the multi-part component kits are shown in **Table 124** through **Table 128**.

Table 123 Kits for SureSelect Max Target Enrichment with Fast or Overnight Hybridization

SureSelect Max Kits and Included Component Kits	Component Kit Part Numbers for 96 Hyb Kit	Storage Condition
SureSelect Max Fast Hyb Kit (G9689B)		
SureSelect Max Target Enrichment Kit Fast Hyb Module, Box 1	5282-0130	Room Temperature
SureSelect Max Target Enrichment Kit Fast Hyb Module, Box 2	5282-0131	–20°C
SureSelect Streptavidin Beads	5191-5742	+4°C
SureSelect Max Overnight Hyb Kit (G9690B)		
SureSelect Max Target Enrichment Kit Overnight Hyb Module, Box 1	5282-0134	Room Temperature
SureSelect Max Target Enrichment Kit Overnight Hyb Module, Box 2	5282-0135	–20°C
SureSelect Streptavidin Beads	5191-5742	+4°C
SureSelect Max Blockers and Primers Module for ILM (G9699B)	5282-0137	–20°C
SureSelect Max Purification Beads (G9962B)	5282-0226	+4°C

Component Kit Details

Table 124 SureSelect Max Target Enrichment Kit Fast Hyb Module, Box 1 content

Kit Component	96 Hyb Kit (p/n 5282-0130)
SureSelect Binding Buffer	bottle
SureSelect Wash Buffer 1	bottle
SureSelect Wash Buffer 2	bottle

Table 125 SureSelect Max Target Enrichment Kit Fast Hyb Module, Box 2 content

Kit Component	96 Hyb Kit (p/n 5282-0131)
SureSelect Max Fast Hyb Buffer	bottle
SureSelect RNase Block	tube with purple cap
Amplification Master Mix	bottle

Table 126 SureSelect Max Target Enrichment Kit Overnight Hyb Module, Box 1 content

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

Table 127 SureSelect Max Target Enrichment Kit Overnight Hyb Module, Box 2 content

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

Table 128 SureSelect Max Blockers and Primers Module for ILM content

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

Index Primer Pair Plate Maps

Plate positions of the SureSelect Max UDI Primers for ILM provided with 96 reaction kits are shown in **Table 129** through **Table 132**.

CAUTION The SureSelect Max UDI Primers are provided in single-use aliquots. To avoid cross-contamination of libraries, use each well in only one library preparation reaction. Do not retain and re-use any residual volume for subsequent experiments.

Table 129 Plate map for SureSelect Max UDI Primers 1-96, provided in orange plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Table 130 Plate map for SureSelect Max UDI Primers 97-192, provided in blue plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Table 131 Plate map for SureSelect Max UDI Primers 193-288, provided in green plate

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

Table 132 Plate map for SureSelect Max UDI Primers 289-384, provided in red plate

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

Quick Reference Tables for Master Mixes and Source Plates

This section contains copies of the tables for master mix formulations and source plate volumes used in the SureSelect Max DNA - Enzymatic Fragmentation - Automated using NGS Workstation protocol.

Enzymatic Fragmentation and Library Preparation

Table 133 Preparation of Frag/A-Tail master mix for EnzFrag_ERA_SS_Max protocol

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Frag/A-Tail Buffer (yellow cap)	4 µL	79.9 µL	117.3 µL	128.0 µL	170.7 µL	255.9 µL	491.3 µL
Frag/A-Tail Enzyme Mix (green cap)	6 µL	119.9 µL	176.0 µL	192.0 µL	256.0 µL	383.9 µL	737.0 µL
Total Volume	**10.0 µL**	**199.8 µL**	**293.3 µL**	**320.0 µL**	**426.7 µL**	**639.8 µL**	**1228.3 µL**

Table 134 Preparation of the master mix source plate for EnzFrag_ERA_SS_Max protocol

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Frag/A-Tail master mix	Column 1 (A1-H1)	23.5 µL	34.5 µL	39.5 µL	52.7 µL	78.5 µL	144.5 µL

Table 135 Preparation of the master mix source plate for AdapLig_Bead_Cleanup_SS_Max_runset

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Ligation Master Mix (bottle)	Column 2 (A2-H2)	34.5 µL	55.5 µL	71.6 µL	95.5 µL	139.5 µL	267.0 µL
SureSelect Max MBC Adaptor Oligo Mix, ILM (white cap) OR SureSelect Max MBC-Free Adaptor Oligo Mix (black cap)	Column 3 (A3-H3)	17.5 µL	22.9 µL	21.6 µL	28.8µL	42.7µL	79.2 µL

Pre-Capture PCR

Table 136 Preparation of the master mix source plate for Pre-CapPCR_SS_Max protocol

Reagent	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Amplification Master Mix	Column 4 (A4-H4)	35.0 µL	60.0 µL	83.0 µL	110.0 µL	160.0 µL	310.0 µL

Table 137 Preparation of the Sample Buffer source plate for TS_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
D1000 Sample Buffer	Column 5 (A5-H5)	11 µL	17 µL	23 µL	29 µL	42 µL	80 µL

Fast Hybridization

Table 138 Preparation of Fast Hybridization master mix for Probes <3 Mb, 8 rows of wells

Target size <3.0 Mb							
Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	4.5 µL	75.3 µL	115.3 µL	144.0 µL	191.9 µL	275.4 µL	533.1 µL
SureSelect RNase Block, undiluted (purple cap)	0.5 µL	8.4 µL	12.8 µL	16.0 µL	21.3 µL	30.6 µL	59.2 µL
SureSelect Max Fast Hyb Buffer (bottle)	6.0 µL	100.4 µL	153.8µL	192.0 µL	255.9 µL	367.2 µL	710.9 µL
Probe (with design <3.0 Mb)	2.0 µL	33.5 µL	51.3 µL	64.0 µL	85.3 µL	122.4 µL	237.0 µL
Total Volume	**13.0 µL**	**217.6 µL**	**333.2 µL**	**416.0 µL**	**554.4 µL**	**795.6 µL**	**1540.2 µL**

Table 139 Preparation of Fast Hybridization master mix for Probes ≥3 Mb, 8 rows of wells, fast hybridization

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	25.1 µL	38.4 µL	48.0 µL	64.0 µL	91.8 µL	177.7 µL
SureSelect RNase Block, undiluted (purple cap)	0.5 µL	8.4 µL	12.8 µL	16.0 µL	21.3 µL	30.6 µL	59.2 µL
SureSelect Max Fast Hyb Buffer (bottle)	6 µL	100.4 µL	153.8 µL	192.0 µL	255.9 µL	367.2 µL	710.9 µL
Probe (with design ≥3.0 Mb)	5 µL	83.7 µL	128.2 µL	160.0 µL	213.2 µL	306.0 µL	592.4 µL
Total Volume	**13.0 µL**	**217.6 µL**	**333.2 µL**	**416.0 µL**	**554.4 µL**	**795.6 µL**	**1540.2 µL**

Table 140 Preparation of Fast Hybridization master mix for Probes <3 Mb, single row of wells, fast hybridization

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	5.8 µL	8.8 µL	11.6 µL	14.9 µL	21.1 µL	40.8 µL
SureSelect RNase Block, undiluted (purple cap)	0.5 µL	0.6 µL	1.0 µL	1.3 µL	1.7 µL	2.3 µL	4.5 µL
SureSelect Max Fast Hyb Buffer (bottle)	6 µL	16.6 µL	25.5 µL	33.4 µL	43.2 µL	60.8 µL	117.8 µL
Probe (with design <3 Mb)	2 µL	2.6 µL	3.9 µL	5.1 µL	6.6 µL	9.4 µL	18.1 µL
Total Volume	**13.0µL**	**25.6 µL**	**39.2 µL**	**51.4 µL**	**66.4 µL**	**93.6 µL**	**181.2 µL**

Table 141 Preparation of Fast Hybridization master mix for Probes ≥3 Mb, single row of wells, fast hybridization

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	1.9 µL	2.9 µL	3.9 µL	5.0 µL	7.0 µL	13.6 µL
SureSelect RNase Block, undiluted (purple cap)	0.5 µL	0.6 µL	1.0 µL	1.3 µL	1.7 µL	2.3 µL	4.5 µL
SureSelect Max Fast Hyb Buffer (bottle)	6 µL	16.6 µL	25.5 µL	33.4 µL	43.2 µL	60.8 µL	117.8 µL
Probe (with design ≥3 Mb)	5 µL	6.4 µL	9.8 µL	12.8 µL	16.6 µL	23.4 µL	45.3 µL
Total Volume	**13.0 µL**	**25.6 µL**	**39.2 µL**	**51.4 µL**	**66.4 µL**	**93.6 µL**	**181.2 µL**

Table 142 Preparation of the master mix source plate for Hyb_SS_Max protocol (fast hybridization)

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Blocker Mix, ILM (blue cap)	Column 1 (A1-H1)	17.0 µL	22.5 µL	21.6 µL	28.8 µL	43.2 µL	83.5 µL
Fast Hybridization master mix	Column 2 (A2-H2)	25.6 µL	39.2 µL	51.4 µL	66.4 µL	93.6 µL	181.2 µL

Overnight Hybridization

Table 143 Preparation of Hybridization Buffer

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Hyb 1 (bottle)	6.63 µL	97.3 µL	158.7 µL	220.2 µL	281.6 µL	404.6 µL	791.2 µL
SureSelect Hyb 2 (red cap)	0.27 µL	4.0 µL	6.5 µL	9.0 µL	11.5 µL	16.5 µL	32.2 µL
SureSelect Hyb 3 (yellow cap)	2.65 µL	38.9 µL	63.4 µL	88.0 µL	112.6 µL	161.7 µL	316.3 µL
SureSelect Hyb 4 (black cap)	3.45 µL	50.6 µL	82.6 µL	114.6 µL	146.6 µL	210.5 µL	411.7 µL
Total Volume	**13.0 µL**	**190.8 µL**	**311.2 µL**	**431.8 µL**	**552.3 µL**	**793.3 µL**	**1551.4 µL**

Table 144 Preparation of Overnight Hybridization master mix for Probes <3 Mb, 8 rows of wells

	Target size <3.0 Mb						
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	62.3 µL	101.7 µL	141.1 µL	180.5 µL	259.3 µL	507.2 µL
SureSelect RNase Block (purple cap)	0.5 µL	6.9 µL	11.3 µL	15.7 µL	20.1 µL	28.8 µL	56.4 µL
Overnight Hybridization Buffer (prepared on page 110)	13 µL	180.1 µL	293.9 µL	407.7 µL	521.6 µL	749.2 µL	1465.2 µL
Probe (with design <3.0 Mb)	2 µL	27.7 µL	45.2 µL	62.7 µL	80.2 µL	115.3 µL	225.4 µL
Total Volume	**20 µL**	**277.0 µL**	**452.1 µL**	**627.2 µL**	**802.4 µL**	**1152.6 µL**	**2254.2 µL**

Table 145 Preparation of Overnight Hybridization master mix for Probes ≥3 Mb, 8 rows of wells

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	20.8 µL	33.9 µL	47.0 µL	60.2 µL	86.4 µL	169.1 µL
SureSelect RNase Block (purple cap)	0.5 µL	6.9 µL	11.3 µL	15.7 µL	20.1 µL	28.8 µL	56.4 µL
Overnight Hybridization Buffer (prepared on **page 110**)	13 µL	180.1 µL	293.9 µL	407.7 µL	521.6 µL	749.2 µL	1465.2 µL
Probe (with design ≥3.0 Mb)	5 µL	69.3 µL	113.1 µL	156.8 µL	200.6 µL	288.2 µL	563.6 µL
Total Volume	**20 µL**	**277.1 µL**	**452.2 µL**	**627.2 µL**	**802.5 µL**	**1152.6 µL**	**2254.2 µL**

Table 146 Preparation of Overnight Hybridization master mix for Probes <3 Mb, single row of wells

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	7.3 µL	12.0 µL	16.6 µL	21.2 µL	30.5 µL	59.7 µL
SureSelect RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.4 µL	3.4 µL	6.6 µL
Overnight Hybridization Buffer (prepared on **page 110**)	13 µL	21.2 µL	34.6 µL	48.0 µL	61.4 µL	88.1 µL	172.4 µL
Probe (with design <3 Mb)	2 µL	3.3 µL	5.3 µL	7.4 µL	9.4 µL	13.6 µL	26.5 µL
Total Volume	**20 µL**	**32.6 µL**	**53.2 µL**	**73.8 µL**	**94.4 µL**	**135.6 µL**	**265.2 µL**

Table 147 Preparation of Overnight Hybridization master mix for Probes ≥3 Mb, single row of wells

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	2.4 µL	4.0 µL	5.5 µL	7.1 µL	10.2 µL	19.9 µL
SureSelect RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.4 µL	3.4 µL	6.6 µL
Overnight Hybridization Buffer (prepared on **page 110**)	13 µL	21.2 µL	34.6 µL	48.0 µL	61.4 µL	88.1 µL	172.4 µL
Probe (with design ≥3 Mb)	5 µL	8.2 µL	13.3 µL	18.5 µL	23.6 µL	33.9 µL	66.3 µL
Total Volume	**20 µL**	**32.6 µL**	**53.2 µL**	**73.8 µL**	**94.5 µL**	**135.6 µL**	**265.2 µL**

Table 148 Preparation of the master mix source plate for Hyb_SS_Max protocol (overnight hybridization)

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Blocker Mix, ILM (blue cap)	Column 1 (A1-H1)	17.0 µL	22.5 µL	21.6 µL	28.8 µL	43.2 µL	83.5 µL
Overnight Hybridization master mix	Column 2 (A2-H2)	32.6 µL	53.2 µL	73.8 µL	94.4 µL	135.6 µL	265.2 µL

Hybrid Capture and Washing

Table 149 SureSelect Streptavidin Beads washing mixture

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Streptavidin Beads	40 µL	340 µL	660 µL	980 µL	1.32 mL	2.0 mL	4.0 mL
SureSelect Binding Buffer	160 µL	1.36 mL	2.64 mL	3.92 mL	5.28 mL	8.0 mL	16.0 mL
Total Volume	**200 µL**	**1.7 mL**	**3.3 mL**	**4.9 mL**	**6.6 mL**	**10 mL**	**20 mL**

Table 150 Preparation of magnetic beads for Capture&Wash_SS_Max runset

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Binding Buffer	200 µL	1.7 mL	3.3 mL	4.9 mL	6.6 mL	10 mL	20 mL

Post-Capture PCR

Table 151 Preparation of Post-Capture PCR Master Mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Amplification Master Mix (bottle)	25 µL	343.3 µL	563.9 µL	709.9 µL	946.3 µL	1419.4 µL	2770.7 µL
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	13.7 µL	22.6 µL	28.4 µL	37.9 µL	56.8 µL	110.8 µL
Total Volume	**26 µL**	**357.0 µL**	**586.5 µL**	**738.3 µL**	**984.2 µL**	**1476.2 µL**	**2881.5 µL**

Table 152 Preparation of the master mix source plate for Post-CapPCR_SS_Max protocol

Master Mix Solution	Position on Source Plate	Volume of master mix added per well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Post-Capture PCR Master Mix	Column 3 (A3-H3)	42.0 µL	69.0 µL	91.2 µL	121.5 µL	177.0 µL	339.0 µL

Table 153 Preparation of the Sample Buffer Source Plate for TS_HighSensitivity_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
High Sensitivity D1000 Sample Buffer	Column 4 (A4-H4)	8 µL	11 µL	14 µL	17 µL	24 µL	44 µL

Quick Reference Tables for Other Reagent Volumes

This section contains tables the summarize the gDNA input volumes, volume of UDI Primers in the primer plate, volumes used for reservoirs of water and ethanol, and volumes of purification beads used in the automation protocols.

Table 154 Genomic DNA Input Volumes

Genomic DNA Input for 1 Library	Volume
10–200 ng	40 µL

Table 155 SureSelect Max UIDI Index Primer Pairs Volume on Primer Plate

Reagent	Volume for 1 Library
SureSelect Max UDI Primers for ILM	5 µL

Table 156 SureSelect Max Purification Beads Volumes for Bead_Cleanup_SS_Max Protocols

Protocol or Runset	Volume of Beads per Well*
AdapLig_Bead_Cleanup_SS_Max_runset	60 µL
Bead_Cleanup_SS_Max (Pre-Cap PCR - SinglePlex)	50 µL
Bead_Cleanup_SS_Max (Pre-Cap PCR - MultiPlex)	50 µL
Bead_Cleanup_SS_Max (Concentration of Pool)	180 µL
Bead_Cleanup_SS_Max (Post-Capture PCR)	50 µL

* When preparing the plates of purification beads, fill the columns of the reservoir with enough of the bead suspension to cover the pyramid-shaped wells

Table 157 Water and Ethanol Volumes for Bead_Cleanup_SS_Max Protocols

Reagent	Volume per Reservoir
70% ethanol in Agilent deep well reservoir	50 mL
Nuclease-free water in Agilent shallow well reservoir	30 mL

Troubleshooting Guide

If recovery of gDNA from samples is low

Using excess tissue for gDNA isolation can reduce yield. Use only the amount of each specific tissue type recommended by the gDNA isolation protocol.

Tissue sample lysis may not have been optimal during gDNA isolation. Monitor the extent of sample lysis during the Proteinase K digestion at 56°C by gently pipetting the digestion reaction every 20–30 minutes, visually inspecting the solution for the presence of tissue clumps. If clumps are still present after the 1-hour incubation at 56°C, add another 10 µL of Proteinase K and continue incubating at 56°C, with periodic mixing and visual inspections, for up to two additional hours. When the sample no longer contains clumps of tissue, move the sample to room temperature until lysis is complete for the remaining samples. Do not over-digest. Individual samples may be kept at room temperature for up to 2 hours before resuming the protocol. Do not exceed 3 hours incubation at 56°C for any sample.

If yield of pre-capture libraries is low

Use only nuclease-free water or 1X Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) where specified in the protocol for sample preparation and dilution steps. Do not substitute with standard TE or other solvents containing >0.1 mM EDTA. Higher concentrations of EDTA inhibit the fragmentation reaction and reduce library yield.

The library preparation 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 library preparation for the sample, increasing the pre-capture PCR cycle number by 1 to 2 cycles. If a high molecular weight peak (>500 bp) is observed in the electropherogram for a sample with low yield, the DNA may be over-amplified. Repeat library preparation for the sample, decreasing the PCR cycle number by 1 to 3 cycles.

DNA isolated from FFPE tissue samples may be over-fragmented or have modifications that adversely affect library preparation processes. Use the Agilent NGS FFPE QC Kit to determine the precise quantity of amplifiable DNA in the sample and allow direct normalization of input DNA amount.

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. Adhere to all bead storage and handling conditions recommended by the manufacturer. Ensure that the beads are kept at room temperature for at least 30 minutes before use. Use freshly-prepared 70% ethanol for each purification procedure.

If pre-capture library fragment size is different than expected in electropherograms

FFPE DNA pre-capture libraries may have a smaller fragment size distribution due to the presence of DNA fragments in the input DNA that are smaller than the optimal fragment size for target enrichment.

If library fragments are longer than expected the gDNA samples may be under-fragmented. Use only nuclease-free water or 1X Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) where specified in the protocol for sample preparation and dilution steps. Do not substitute with standard TE or other solvents containing >0.1 mM EDTA. Higher concentrations of EDTA can inhibit DNA fragmentation.

If library fragments are shorter than expected the gDNA samples may be over-fragmented. Make sure to keep DNA samples on ice while setting up the EnzFrag_ERA_SS_Max protocol.

If low molecular weight adaptor-dimer peak is present in pre-capture library electropherograms

The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield of pre-capture libraries.

If yield of post-capture 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

Yield from the magnetic bead purification step may be suboptimal. Adhere to all bead storage and handling conditions recommended by the manufacturer. Ensure that the beads are kept at room temperature for at least 30 minutes before use. Use freshly-prepared 70% ethanol for each purification procedure.

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

If post-capture library fragment size is different than expected in electropherograms

DNA fragment size selection during purification depends upon using the correct ratio of sample to purification beads. When preparing the purification beads plate for post-capture purification, mix the beads until the suspension appears homogeneous and consistent in color before adding the bead suspension to the shallow well reservoir. After preparation of the bead plate, seal the plate and store at 4°C until needed.

If low % on-target is observed in library sequencing results

Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a thermal cycler in close proximity to the NGS Workstation to retain the sample temperature during transfer step (**step 15** on **page 104** for fast hybridization; **step 15** on **page 116** for overnight hybridization).

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 108** on page 147). 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 21** on page 146); failure to remove MBC sequences from both strands may affect alignment quality.

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In This Book

This guide contains information to run the SureSelect Max DNA protocol for library prep (with enzymatic fragmentation) and target enrichment, with optional pre-capture pooling, using automation protocols provided with the Agilent Bravo NGS Workstation (Option B).

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