SureSelect XT HS2 DNA System

Automated using Agilent NGS Bravo Option A

DNA Library Preparation, Pre-Capture Pooling (optional), and Target Enrichment for the Illumina Platform

Protocol

Version A1, September 2022

SureSelect platform manufactured with Agilent SurePrint technology.

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

Notices

Manual Part Number

G9985-90020

Edition

Version A1, September 2022

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Acknowledgment

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CAUTION

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In this Guide...

This guide provides an optimized protocol for preparation of target-enriched Illumina paired-end multiplexed sequencing libraries using SureSelect XT HS2 Reagent Kits. Sample processing steps are automated using the Agilent NGS Bravo Option A.

What's New in Version A1

- Design ID information added to **Table 4** on page 15 for pre-designed SureSelect probes.
- Support for use of Agilent's Alissa Reporter software for SureSelect XT HS2 DNA library sequence pre-processing and human germline DNA variant analysis (see **page 135**).
- Updates to downstream sequencing support information (see **page 136**). Key updates include support for the new CReaK tool, replacing the LocatIt tool in AGeNT v3.0 (see **page 136**), and instructions for MBC trimming using BCL Convert software (see *Note* on **page 136**).
- Updated **"Notice to Purchaser."**
- Updates to post-capture library fragment size range guidelines for libraries prepared using intact input DNA in **Table 77** on page 122.
- New instructions on labeling and sealing the AMPure XP bead plates. See **Chapter 3**, "Preparation of AMPure XP Bead Plates," starting on page 35.

Content

1 Before You Begin

Make sure you have the most current protocol. Go to **www.agilent.com** and search for G9985-90020.

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

NOTE

This protocol describes automated DNA sample processing for SureSelect XT HS2 Target Enrichment using the Agilent NGS Bravo Option A.

For automated sample processing procedures using Agilent NGS Workstation Option B, see publication G9985-90010.

For non-automated sample processing procedures, see publication G9983-90000 (for post-capture pooling workflow) or G9985-90000 (for pre-capture pooling workflow).

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 Agilent NGS Bravo 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.
- 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.
- 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

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

Materials Required

Materials required to complete the SureSelect XT HS2 automation protocol will vary based on the following considerations:

- DNA sample type: high-quality gDNA derived from fresh samples vs. FFPE-derived gDNA samples
- DNA fragmentation method used in workflow: enzymatic fragmentation vs. mechanical (Covaris-mediated) shearing
- Pooling of DNA libraries for multiplexed sequencing: Pre-capture pooling vs. post-capture pooling

To determine the materials required for your unique needs, refer to the tables provided in this chapter.

- See **Table 1** and **Table 2** for the reagents and equipment required for all sample types and workflows.
- See **Table 3** for the SureSelect XT HS2 DNA Reagent Kits. The table includes kits that are suitable for workflows with pre-capture pooling of DNA libraries and kits suitable for workflows with post-capture pooling of DNA libraries.
- See **Table 4** for compatible probes. The table distinguishes between probes suitable for workflows with pre-capture pooling of DNA libraries and probes suitable for workflows with post-capture pooling of DNA libraries.
- See **Table 5** for additional materials needed to complete the protocols using the selected DNA sample type and fragmentation method.
- See **Table 6** for a list of optional materials.

Table 1 Required Reagents--All Sample and Workflow Types

Description	Vendor and part number
AMPure XP Kit* 60 mL 450 mL	Beckman Coulter Genomics p/n A63881 p/n A63882
Dynabeads MyOne Streptavidin T1* 10 mL 50 mL	Thermo Fisher Scientific p/n 65602 p/n 65604D
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 100 assays 500 assays	Thermo Fisher Scientific p/n Q32850 p/n Q32853
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

* Separate purchase **not** required when using the SureSelect XT HS2 DNA Reagent Kits that include SureSelect DNA AMPure XP Beads and SureSelect Streptavidin Beads (Agilent p/n G9984A, G9984B, G9984C, or G9984D).

Table 2 Required Equipment--All Sample and Workflow Types

Description	Vendor and Part Number
Agilent NGS Bravo Option A Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5573AA (VWorks software version 13.1.0.1366)
Agilent PlateLoc Thermal Microplate Sealer	Agilent p/n G5585HA or G5585BA
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
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 Agilent NGS Bravo. 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 Agilent NGS Bravo 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 or 951020619 • 96 Armadillo PCR plates (full-skirted), Thermo Fisher Scientific p/n AB2396
Eppendorf twin.tec full-skirted 96-well PCR plates, or Armadillo PCR plates, 96-wells (full-skirted)	Eppendorf p/n 951020401 or 951020619 Thermo Fisher Scientific p/n AB2396
Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 19 mm height – used when Bravo 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 Bravo setup calls for **Agilent Deep Well Reservoir**	Agilent p/n 201244-100
Agilent Storage/Reaction Microplates, 96 wells, 1 mL/round well – used when Bravo setup calls for **Agilent DeepWell Plate** or **Agilent DW Plate**	Agilent p/n 203426-100
Agilent Storage/Reaction Microplates, 96 wells, 2 mL/square well – used when Bravo setup calls for **Waste Plate (Agilent 2 mL Square Well)**	Agilent p/n 201240-100
Nucleic acid surface decontamination wipes	DNA Away Surface Decontaminant Wipes, Thermo Scientific p/n 7008, or equivalent
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

Table 2 Required Equipment--All Sample and Workflow Types (continued)

Description	Vendor and Part Number
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
DNA Analysis Platform and Consumables*	
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
OR	
Agilent 4200 TapeStation†	Agilent p/n G2991AA
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
D1000 Sample Buffer‡	Agilent p/n 5067-5602
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585
High Sensitivity D1000 Sample Buffer‡	Agilent p/n 5067-5603

* DNA samples may also be analyzed using the Agilent 5200 Fragment Analyzer, p/n M5310AA, and associated NGS Fragment Kits (e.g., DNF-473-0500 and DNF-474-0500). Follow the assay instructions provided for each NGS Fragment Kit.

† DNA samples may also be analyzed using the Agilent 4150 TapeStation, p/n G2992AA. ScreenTape devices plus their associated reagent kits, and the 8-well strip tubes and caps listed in this table are compatible with both platforms.

‡ The additional TapeStation assay sample buffers, p/n 5067-5602 and 5067-5603, are required only when sample qualification is performed using VWorks automation protocols TS_D1000 and TS_HighSensitivity_D1000.

Table 3 Agilent SureSelect XT HS2 DNA Reagent Kits

Description	Agilent Part Number
For *Pre-Capture* Pooling	
SureSelect XT HS2 DNA Library Preparation Kit for ILM (Pre PCR), 96 Reactions	G9985A (with Index Pairs 1–96) G9985B (with Index Pairs 97–192) G9985C (with Index Pairs 193–288) G9985D (with Index Pairs 289–384)
AND	
SureSelect XT HS2 DNA Target Enrichment Kit (Post PCR), 12 Hybs*	G9987A
For *Post-Capture* Pooling	
SureSelect XT HS2 DNA Reagent Kit, 96 Reactions *-- contains reagents for library preparation and target enrichment*	G9983A (with Index Pairs 1–96) G9983B (with Index Pairs 97–192) G9983C (with Index Pairs 193–288) G9983D (with Index Pairs 289–384)
OR	
SureSelect XT HS2 DNA Reagent Kit with AMPure XP/Streptavidin Beads, 96 Reactions *-- contains reagents for library preparation and target enrichment, and includes the necessary AMPure XP and Streptavidin beads*	G9984A (with Index Pairs 1–96) G9984B (with Index Pairs 97–192) G9984C (with Index Pairs 193–288) G9984D (with Index Pairs 289–384)

* The 12-Hyb Target Enrichment Kit provides sufficient reagents for 12 hybridization reactions of pre-capture pooled samples, which corresponds to 1.5 columns (i.e., 12 wells) on a 96-well plate. The hybridization protocol cannot process partial columns. Purchase two 12-Hyb Target Enrichment Kits to run 3 full columns (i.e., 24 wells) of hybridization reactions. If your workflow calls for hybridization of 96 pooled samples, then purchase eight of the 12-Hyb Target Enrichment Kits. Pre-capture pooling of samples can use the configuration of either 8 samples/pool or 16 samples/pool.

Table 4 Compatible Probes based on Pooling Method

Probe Capture Library		Design ID	Ordering Information
Custom Probes*			
***Pre-Capture* Pooling**	SSEL PreCap Custom Tier1 1–499 kb (6 Hybs† or 30 Hybs‡)	Please visit the SureDesign website to design Custom SureSelect probes and obtain ordering information. Contact the SureSelect support team (see **page 2**) or your local representative if you need assistance.	
	SSEL PreCap Custom Tier2 0.5 –2.9 Mb (6 Hybs† or 30 Hybs‡)		
	SSEL PreCap Custom Tier3 3 –5.9 Mb (6 Hybs† or 30 Hybs‡)		
	SSEL PreCap Custom Tier4 6 –11.9 Mb (6 Hybs† or 30 Hybs‡)		
	SSEL PreCap Custom Tier5 12–24 Mb (6 Hybs† or 30 Hybs‡)		
For *Post-Capture* Pooling	SureSelect Custom Tier1 1–499 kb		
	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		
Pre-designed Probes			
***Pre-Capture* Pooling**	SureSelect XT HS PreCap Human All Exon V8 (12 Hybs)**	S33266340	Agilent p/n 5191-6878
	SureSelect XT HS PreCap Human All Exon V8+UTR (12 Hybs)**	S33613271	Agilent p/n 5191-7406
	SureSelect XT HS PreCap Human All Exon V8+NCV (12 Hybs)**	S33699751	Agilent p/n 5191-7412
	SureSelect Pre-Capture Pooling Human All Exon V7 (12 Hybs)**	S31285117	Agilent p/n 5191-5735
	SureSelect XT2 Clinical Research Exome V2 (12 Hybs)**	S30409818	Agilent p/n 5190-9501
	SureSelect XT2 Mouse All Exon (12 Hybs)**	S0276129	Agilent p/n 5190-4682
	ClearSeq Inherited Disease XT2 (12 Hybs)**	S0684402	Agilent p/n 5190-7525
	ClearSeq Comprehensive Cancer XT2 (6 Hybs)†	0425761	Agilent p/n 5190-8018
***Post-Capture* Pooling**	SureSelect XT HS Human All Exon V8	S33266340	Agilent p/n 5191-6875
	SureSelect XT HS Human All Exon V8+UTR	S33613271	Agilent p/n 5191-7403
	SureSelect XT HS Human All Exon V8+NCV	S33699751	Agilent p/n 5191-7409
	SSel XT HS and XT Low Input Human All Exon V7, 96 Reactions	S31285117	Agilent p/n 5191-4029
	SureSelect XT Clinical Research Exome V2, 96 Reactions	S30409818	Agilent p/n 5190-9492
	SureSelect XT Mouse All Exon, 96 Reactions	S0276129	Agilent p/n 5190-4642
	ClearSeq Comprehensive Cancer XT, 96 Reactions	0425761	Agilent p/n 5190-8012
	ClearSeq Inherited Disease XT, 96 Reactions	S0684402	Agilent p/n 5190-7519
Pre-designed Probes customized with additional *Plus* custom content			
***Pre-Capture* Pooling**	SureSelect XT2 Clinical Research Exome V2 Plus 1 (12 Hybs)**	Please visit the SureDesign website to design the customized *Plus* content and obtain ordering information. Contact the SureSelect support team (see **page 2**) or your local representative if you need assistance.	
	SureSelect XT2 Clinical Research Exome V2 Plus 2 (12 Hybs)**		
	ClearSeq Comprehensive Cancer Plus XT2 (6 Hybs)†		
	ClearSeq Inherited Disease Plus XT2 (12 Hybs)**		
***Post-Capture* Pooling**	SSel XT HS and XT Low Input Human All Exon V7 Plus 1		
	SSel XT HS and XT Low Input Human All Exon V7 Plus 2		
	SureSelect XT Clinical Research Exome V2 Plus 1		
	SureSelect XT Clinical Research Exome V2 Plus 2		
	ClearSeq Comprehensive Cancer Plus XT		
	ClearSeq Inherited Disease Plus XT		

* Custom Probes designed August 2020 or later are produced using an updated manufacturing process; design-size Tier is shown on labeling for these products. Custom Probes designed and ordered prior to August 2020 may be reordered, with these probes produced using the legacy manufacturing process; design-size Tier is not shown on labeling for the legacy-process products. Custom Probes of both types use the same optimized target enrichment protocols detailed in this publication.

† 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). Contains enough reagent for 6 hybridization reactions using the run setup on **page 96**. The hybridization protocol can only process columns in which all 8 wells contain a hybridization reaction. 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 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). Contains enough reagent for 30 hybridization reactions using the run setup on **page 96**. The hybridization protocol can only process columns in which all 8 wells contain a hybridization reaction.

** 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). Contains enough reagent for 6 hybridization reactions using the run setup on **page 96**. The hybridization protocol can only process columns in which all 8 wells contain a hybridization reaction. 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.

Table 5 Additional Required Materials based on DNA Sample Type/Fragmentation Method

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
Required for enzymatic fragmentation of DNA samples (not required for workflows with mechanical shearing)	
SureSelect Enzymatic Fragmentation Kit, 96 Reactions Automation	Agilent p/n 5191-6764
Required for mechanical shearing of DNA samples (not required for workflows with enzymatic fragmentation)	
Covaris Sample Preparation System	Covaris model E220
Covaris 96 microTUBE plate	Covaris p/n 520078

Optional Materials

Table 6 Supplier Information for Optional Materials

Description	Vendor and part number
High-quality gDNA purification system, for example: QIAamp DNA Mini Kit 50 Samples 250 Samples	 Qiagen p/n 51304 p/n 51306
Tween 20	Sigma-Aldrich p/n P9416-50ML

2 Using the Agilent NGS Bravo Option A for SureSelect Target Enrichment

This chapter contains an orientation to the Agilent NGS Bravo Option A, an overview of the SureSelect XT HS2 target enrichment protocol, and considerations for designing SureSelect XT HS2 experiments for automated processing using the Agilent NGS Bravo Option A.

About the NGS Bravo Option A

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 and software. Refer to the user guides listed in **Table 7**.

Review the user guides listed in **Table 7** (available at Agilent.com) to become familiar with the general features and operation of the automation components. Instructions for using the Bravo platform and other automation components in the SureSelect XT HS2 Target Enrichment workflow are detailed in this user guide.

Table 7 Agilent NGS Bravo User Guide reference information

Device	User Guide part number
Bravo Platform	SD-V1000376 (previously G5562-90000)
VWorks Software (version 13.1.0.1366)	G5415-90068
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.

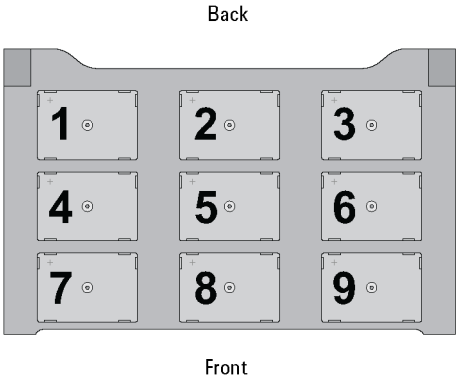


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

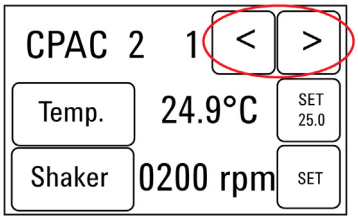
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the 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 8** for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

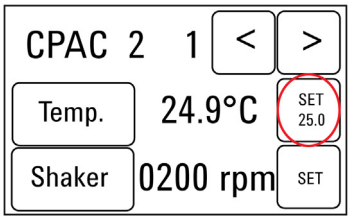
Table 8 Inheco Multi TEC Control touchscreen designations

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

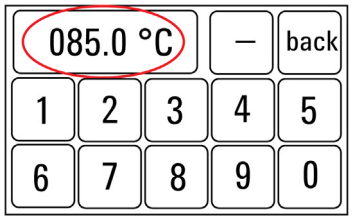
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



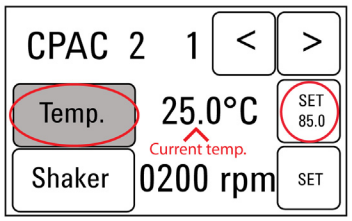
2 To set the temperature of the selected block, press the SET button.



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 Agilent NGS Bravo Option A, allows you to control the integrated devices using a PC. The Agilent NGS Bravo Option A 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 is 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 13.1.0.1366.

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

Logging in to the VWorks software

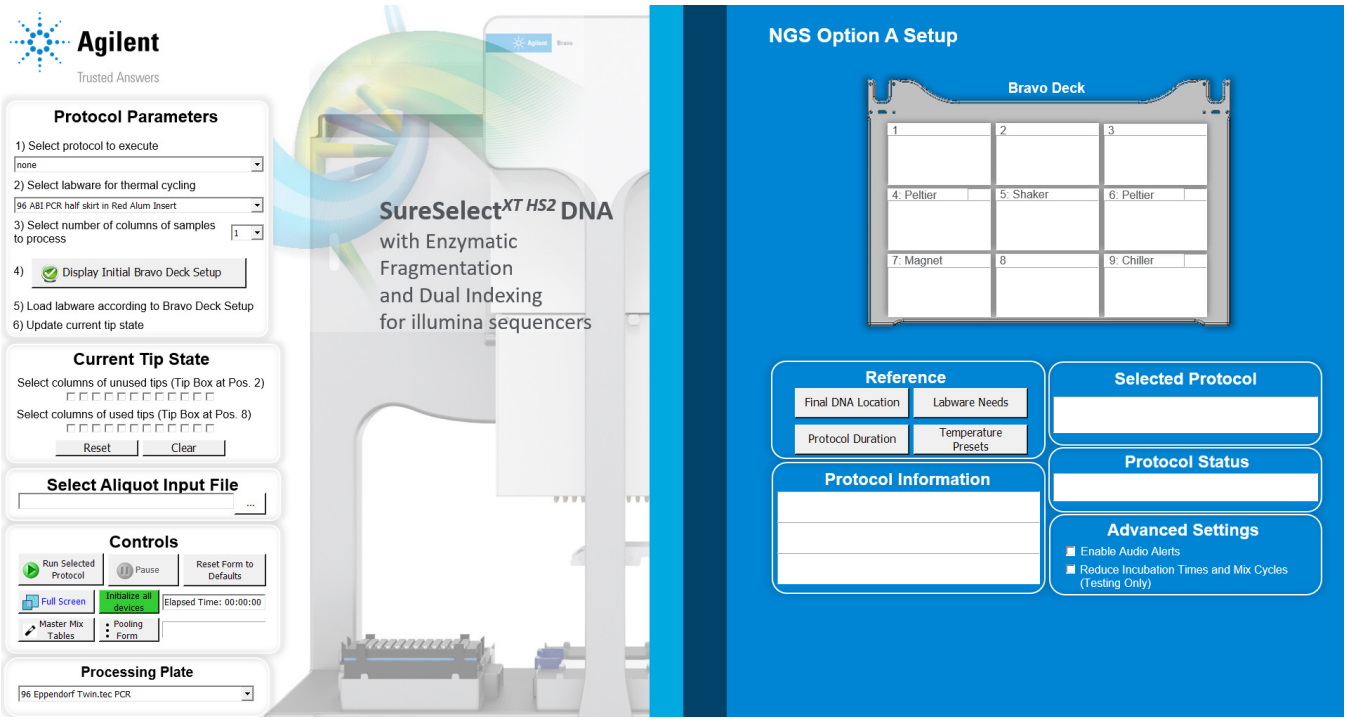
1 Double-click the VWorks icon or the XT_HS2_ILM_v.A1.0.2.VWForm shortcut on the Windows desktop to start the VWorks software.

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.)

Using the XT_HS2_ILM_v.A1.0.2.VWForm to setup and start a run

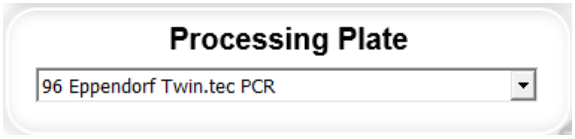
Use the VWorks form XT_HS2_ILM_v.A1.0.2.VWForm, shown below, to set up and start each SureSelect automation protocol.



1 Open the form using the XT_HS2_ILM_v.A1.0.2.VWForm shortcut on your desktop.

2 Verify that the **Processing Plate** selection is set to the correct plate type.

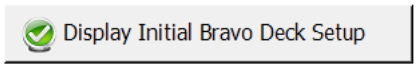
The processing plate is either a 96-well Eppendorf twin.tec plate (Eppendorf p/n 951020401 or 951020619) or a 96-well Armadillo plate (Thermo Fisher Scientific p/n AB2396).



CAUTION Indicating the correct processing plate type that will be used in the assay is critical for accurate pipetting and to avoid damage to the Bravo instrument.

3 Use the drop-down menus on the form to select the appropriate SureSelect workflow step and other Parameters for the run.

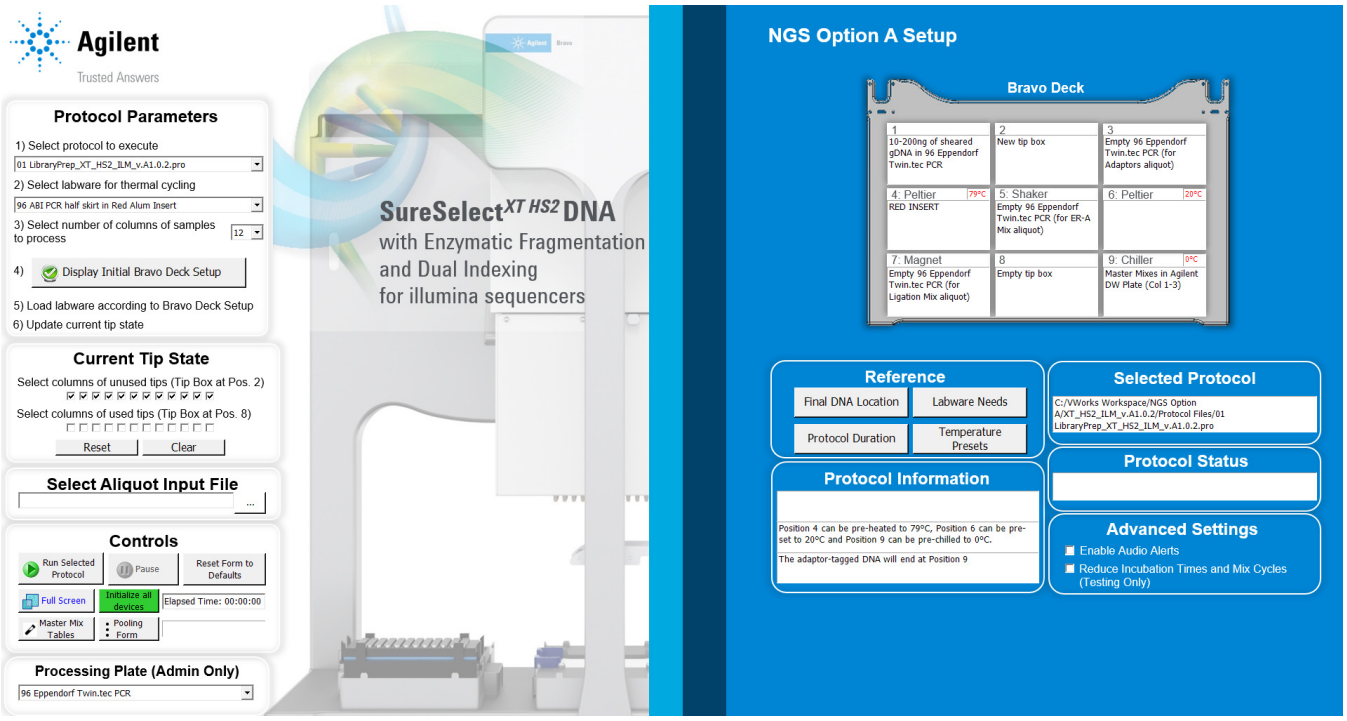
4 Once all run parameters have been specified on the form, click **Display Initial Bravo Deck Setup**.



5 The form will then display the NGS Bravo deck configuration needed for the specified run parameters.

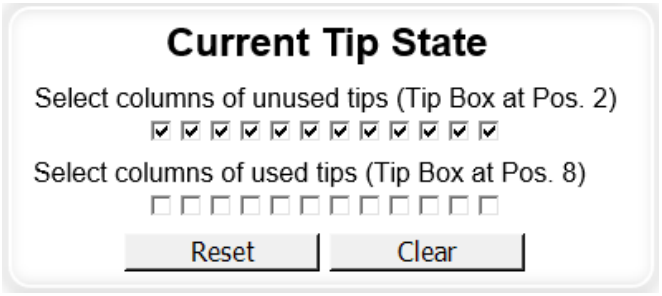
Load the Bravo Deck with labware and reagents as specified in the **Bravo Deck** region of the form.

Review the temperature preset and in-run labware transfer information shown in the **Protocol Information** section of the form. Set the temperature of Bravo Deck positions as needed.



6 Verify that the **Current Tip State** indicator on the form (shown below) matches the configuration of unused tips in the tip box at Bravo Deck position 2.

For a fresh tip box, containing 12 columns of tips, all positions of the **Current Tip State** unused tip indicator (top portion, Box 2) should be selected, as shown below. Clicking **Reset** selects all columns for position 2.



Also verify that the used tip indicator (bottom portion, Box 8) matches the configuration of used tips in the tip box at Bravo Deck position 8.

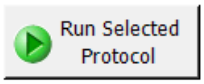
For an empty tip box, all positions of the **Current Tip State** used tip indicator (bottom portion, Box 8) should be cleared, as shown above. Clicking **Reset** clears all columns for position 8.

NOTE

It is important that the **Current Tip State** indicator matches the configuration of tips present at Bravo Deck positions 2 and 8 when initiating the run. Tips that are inappropriately loaded onto the Bravo platform pipette head, or tips missing from the pipette head, will interfere with automated processing steps.

You can use partial tip boxes for NGS Bravo Option A automation protocols, as long as positions of available tips are accurately indicated during run setup.

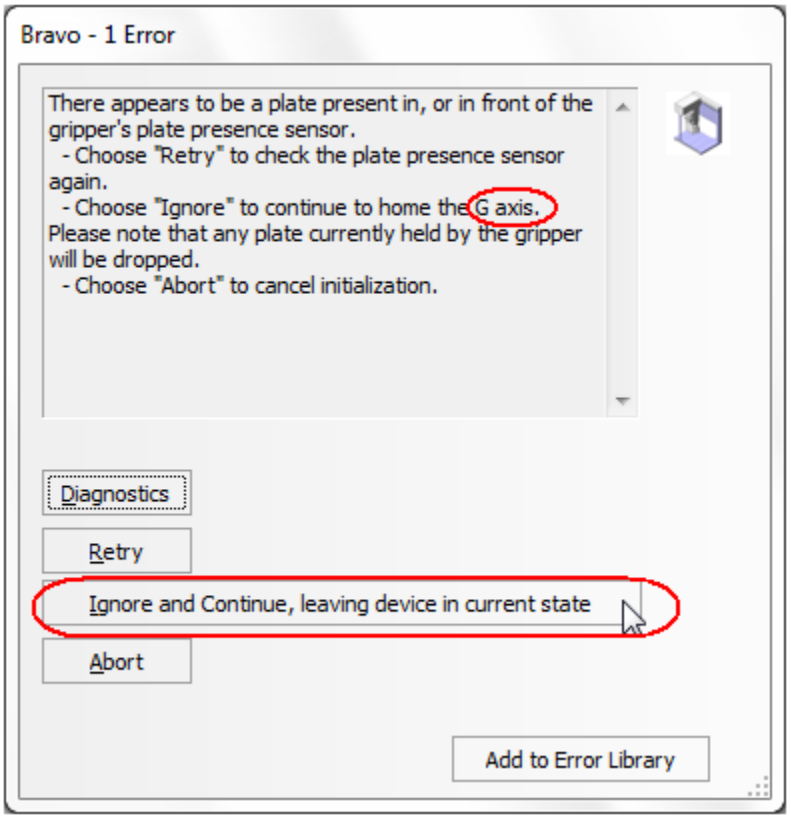
7 After verifying that the NGS Bravo has been set up correctly, click **Run Selected Protocol**.



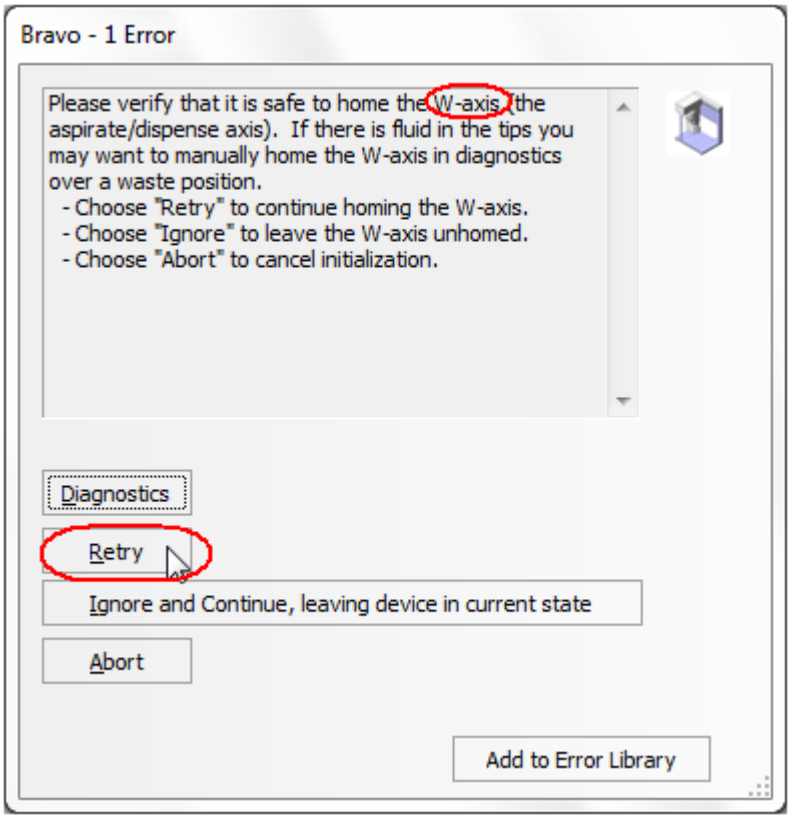
Error messages encountered at start of run

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 Bravo or your run setup.

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



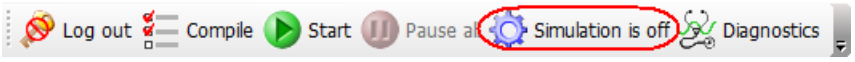
2 If you encounter the W-axis error message shown below, select **Retry**.



Verifying the Simulation setting

VWorks software may be run in simulation mode, during which commands entered on screen are not completed by the NGS Bravo. If NGS Bravo 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 **View > 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 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.

Overview of the SureSelect Target Enrichment Procedure

Figure 2 summarizes the SureSelect XT HS2 library preparation and target enrichment workflow for DNA samples to be sequenced using the Illumina paired-read sequencing platform. For each sample to be sequenced, individual library preparations, hybridizations, and captures are performed.

Agilent offers four different plates of index pairs for use with the SureSelect XT HS2 DNA library preparation reagents to allow for multiplexed sequencing (refer to **"Index Primer Pair Plate Maps"** on page 152). Depending on the SureSelect XT HS2 DNA Reagent Kit(s) used in the protocol, 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 of AMPure XP Bead Plates**, **Sample Preparation**, **Hybridization**, and **Post-Capture Sample Processing for Multiplexed Sequencing** chapters for complete instructions for use of the VWorks protocols for sample processing.

The SureSelect XT HS2 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.

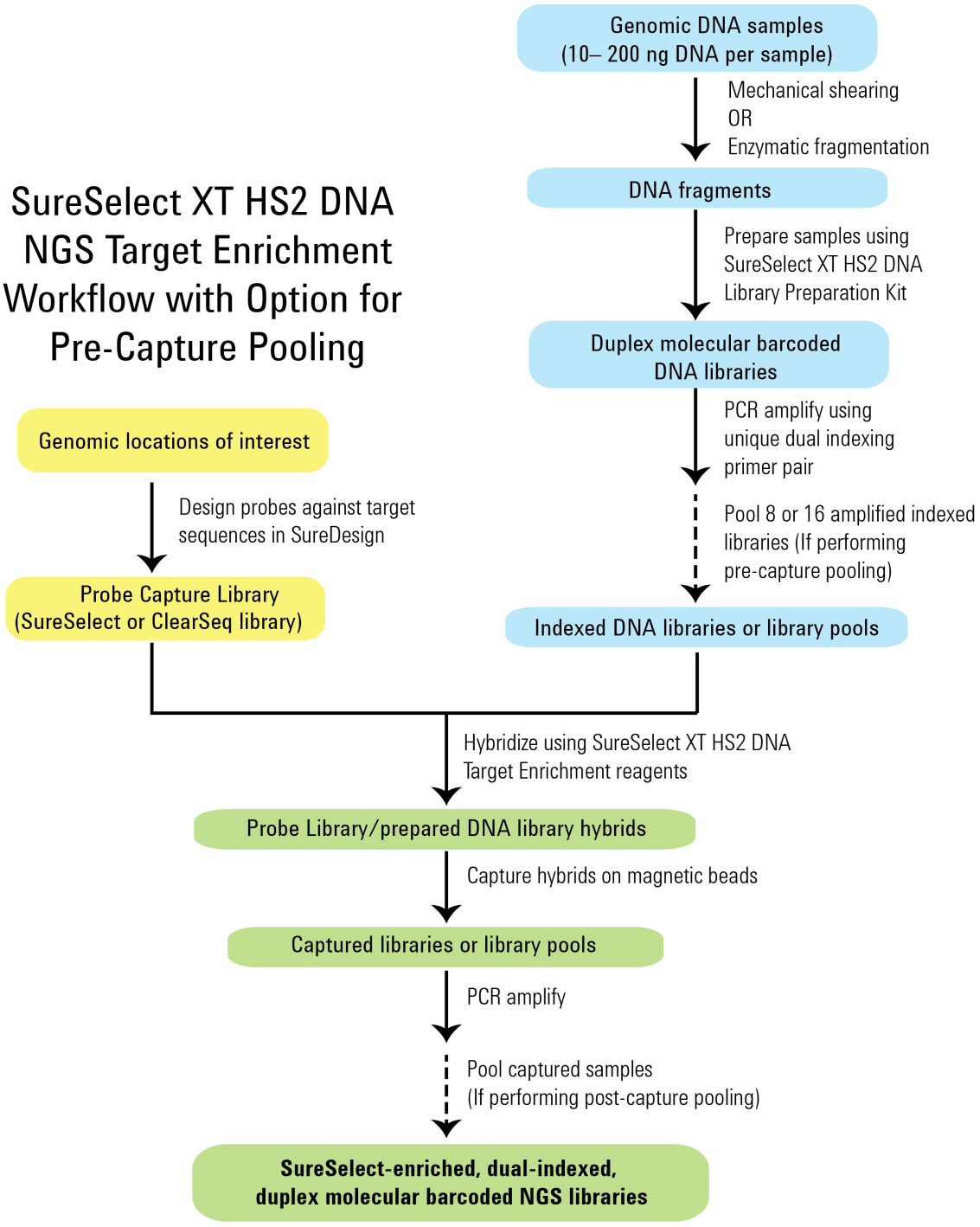


Figure 2 Overall sequencing sample preparation workflow.

Workflow Modulations

The SureSelect XT HS2 DNA target enrichment workflow can be modulated for different applications as described below and summarized in **Table 9** on page 29.

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.

DNA Fragmentation Method Automated Enzymatic Fragmentation protocols are provided to support enzymatic fragmentation with automated liquid handling steps (see **"Method 1: Prepare fragmented DNA by enzymatic fragmentation"** on page 48). Alternatively, DNA can be mechanically sheared using manual liquid handling steps without automation (see **"Method 2. Prepare fragmented DNA by mechanical shearing"** on page 54).

Sample Pooling Options The automated SureSelect XT HS2 DNA target enrichment workflow supports two different approaches for sample pooling, with each using different SureSelect XT HS2 DNA reagents.

- Pre-capture pooling – Following PCR amplification of the indexed DNA libraries, pool either 8 or 16 of the libraries (depending on Probe design size) prior to hybridization with the Probe. Each library pool is then hybridized with the Probe.
- Post-capture pooling – Following hybridization 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.

Table 9 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 **"Protocol modifications for FFPE Samples"** on page 138 for summary of protocol modifications.
DNA Fragmentation Method	Enzymatic Fragmentation	Use the Enzymatic Fragmentation automation protocols to perform enzymatic fragmentation of DNA samples. The protocol 00a EnzFrag_XT_HS2_ILM executes the liquid handling steps for the enzymatic fragmentation reactions. The protocol 00b EnzFrag_Dil_XT_HS2_ILM directs the dilution of the fragmented samples to the concentration needed for library preparation. Requires purchase of SureSelect Enzymatic Fragmentation Kit, 96 Reactions Automation (Agilent p/n 5191-6764)
	Mechanical (Covaris) Shearing	Perform mechanical shearing on Covaris Sample Preparation System using manual liquid handling (no automated protocol). Requires purchase of Covaris Sample Preparation System and consumables (see **Table 5**, "Additional Required Materials based on DNA Sample Type/Fragmentation Method," on page 16).
Pooling Strategy	Pre-Capture Pooling	For library preparation, use a SureSelect XT HS2 DNA Library Preparation Kit that is compatible with pre-capture pooling (e.g., Agilent part numbers G9985A through G9985D). For target enrichment, use the SureSelect XT HS2 DNA Target Enrichment Kit (Post PCR). Refer to **Table 3** on page 14.
	Post-Capture Pooling	Use one of the SureSelect XT HS2 DNA Reagent Kits compatible with post-capture pooling (Agilent part numbers G9983A through G9983D, or G9984A through G9984D). Kits include reagents for both library preparation and target enrichment. Refer to **Table 3** on page 14.

Automation Protocols used in the Workflow

Table 10 Overview of VWorks protocols

Workflow Step	Substep	VWorks Protocols Used for NGS Bravo automation
AMPure XP Bead Aliquoting	Aliquot AMPure XP beads for use in the Library Prep protocol	AMPureXP_Aliquot_LibPrep
	Aliquot AMPure XP beads for use in the Pre-Capture PCR purification protocol	AMPureXP_Aliquot_PreCap
	Aliquot AMPure XP Beads for use in the Pre-Capture Pooling protocol for concentrating the DNA	AMPureXP_Aliquot_Pooling
	Aliquot AMPure XP Beads for use in the Post-Capture PCR purification protocol	AMPureXP_Aliquot_PostCap
Enzymatic DNA Fragmentation*	Shear DNA samples using enzymatic fragmentation	00a EnzFrag_XT_HS2_ILM
	Dilute fragmented samples to appropriate concentration	00b EnzFrag_Dil_XT_HS2_ILM
Library Preparation	Prepare duplex, molecular-barcoded DNA libraries	01 LibraryPrep_XT_HS2_ILM
	Purify DNA libraries using AMPure XP beads	02 Cleanup_LibPrep_XT_HS2_ILM
	Amplify indexed DNA libraries with unique dual indexing primer pair	03 Pre-CapPCR_XT_HS2_ILM
	Purify indexed DNA libraries using AMPure XP beads using an elution volume suitable for **single-plexed** hybridization (i.e., the post-capture pooling workflow)	04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM
	Purify indexed DNA libraries using AMPure XP beads using an elution volume suitable for **multi-plexed** hybridization (i.e., the pre-capture pooling workflow)	04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM
	Analyze indexed DNA libraries using Agilent TapeStation platform	05 TS_D1000
Library Pooling (for **pre-capture pooling** workflow)	Pool indexed DNA libraries in pools of 8 or 16	PreCapture_Pooling *This protocol is set up and executed from the XT_HS2_Pooling VWorks Form*
Single-Plex Pre-Hybridization (for **post-capture pooling** workflow)	Aliquot 500-1000 ng of prepped libraries	06a Aliquot_Libraries
Multi-Plex Pre-Hybridization (for **pre-capture pooling** workflow)	Dilute pooled samples of indexed DNA libraries to normalize volumes to 100 µL	06b Aliquot_Water
	Concentrate pooled samples to 24 µL for hybridization	06c PoolingConcentration_XT_HS2_ILM
Hybridization and Capture	Hybridize prepped libraries or library pools (target enrichment)	07 Hyb_XT_HS2_ILM
	Capture DNA hybrids	08 SSELCapture_XT_HS2_ILM
	Wash DNA hybrids	09 SSELWash_XT_HS2_ILM

Table 10 Overview of VWorks protocols

Workflow Step	Substep	VWorks Protocols Used for NGS Bravo automation
Post-Capture Sample Processing	Amplify target-enriched libraries or library pools	10 Post-CapPCR_XT_HS2_ILM
	Purify enriched, amplified libraries or library pools using AMPure XP beads	11 Cleanup_Post-CapPCR_XT_HS2_ILM
	Analyze final libraries or library pools using Agilent TapeStation platform	12 TS_HighSensitivity_D1000
	For post-capture pooling workflow, pool indexed DNA libraries	13 Aliquot_Captures

* To shear DNA samples mechanically, rather than enzymatically, perform liquid handing steps manually on the Covaris Sample Preparation System (see **"Method 2. Prepare fragmented DNA by mechanical shearing"** on page 55). The XT HS2 VWorks Form does not include an automation protocol for mechanical shearing.

Experimental Setup Considerations for Automated Runs

Agilent SureSelect Automated Library Prep and Capture System runs may include 1 to 12 columns (equivalent to 8 to 96 wells) of gDNA samples to be enriched for sequencing on the Illumina platform. Plan your experiments using complete columns of samples.

Table 11 Columns to Samples Equivalency

Number of Columns Processed	Total Number of Samples Processed
1	8
2	16
3	24
4	32
5	40
6	48
7	56
8	64
9	72
10	80
11	88
12	96

The number of columns or samples that may be processed using the supplied reagents 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

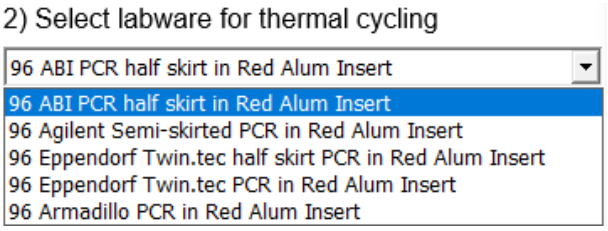
- The NGS Bravo processes samples column-wise beginning at column 1. gDNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- Samples are indexed during pre-capture amplification (see **Figure 2**). Assign each sample to the appropriate indexing primer during experimental design, and place the sample in the well corresponding to its assigned primer. See **Table 99** on page 152 through **Table 102** on page 153 for indexing primer plate maps.
- At the hybridization step (see **Figure 2**), you can add a different SureSelect or ClearSeq Capture Library to each row of the plate. Plan your experiment such that each prepared DNA library plate position corresponds to the appropriate Capture Library row in the sample plate.
- For post-capture amplification (see **Figure 2**), different Capture Libraries can require different amplification cycle numbers, based on sizes of the captured targets. It is most efficient to process similar-sized Capture Libraries on the same plate. See **Table 69** on page 113 to determine which Capture Libraries 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 Bravo to allow rapid and efficient plate transfer.
- Several workflow steps require that the sample plate be sealed using the PlateLoc thermal microplate sealer and then centrifuged to collect any dispersed liquid. To maximize efficiency, locate the centrifuge in close proximity to the NGS Bravo.

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 12**.



CAUTION

The plates listed in **Table 12** are compatible with the Agilent NGS Bravo and associated VWorks automation protocols, designed to support use of various thermal cyclers.

Do not use PCR plates that are not listed in **Table 12**, even if they are compatible with your chosen thermal cycler.

Table 12 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
96 Armadillo PCR plates (full-skirted),	Thermo Fisher Scientific p/n AB2396

3 Preparation of AMPure XP Bead Plates

This chapter provides instructions on preparing all of the plates of AMPure XP beads that are needed throughout the entire workflow. Each plate of AMPure XP beads is prepared using a separate automation protocol available in the XT HS2 VWorks form.

Preparing the plates of AMPure XP 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 AMPure XP beads that are to be used within the day and the following day. *Do not prepare AMPure XP 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.

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

The **02 Cleanup_LibPrep_XT_HS2_ILM** protocol requires a bead plate containing 80 µL of beads in each well. Use the **AMPureXP_Aliquot_LibPrep** protocol to prepare the bead plate needed for library preparation.

Prepare the NGS Bravo and reagents for the AMPureXP_Aliquot_LibPrep protocol

1 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.

2 Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.

- **a** Verify that the AMPure XP bead 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 **02 Cleanup_LibPrep_XT_HS2_ILM** protocol (each column accommodates 8 gDNA samples).

Load the NGS Bravo

3 Load the Bravo deck according to **Table 13**.

Table 13 Initial Bravo deck configuration for AMPureXP_Aliquot_LibPrep protocol

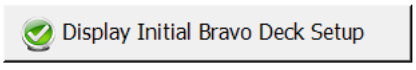
Location	Content
2	New tip box
5	Empty Agilent Deep Well plate
6	Reservoir of AMPure XP bead suspension prepared in **step 2**
8	Empty tip box

Run VWorks protocol AMPureXP_Aliquot_LibPrep

4 On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot_LibPrep** protocol.

5 Select the number of columns of samples to be processed.

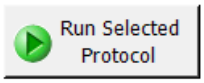
6 Click **Display Initial Bravo Deck Setup**.



7 Verify that the Bravo deck has been set up as displayed on the right side of the form.

8 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **AMPureXP_Aliquot_LibPrep** protocol takes approximately 5 minutes. The protocol directs the NGS Bravo to aliquot 80 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent Deep Well plate.

10 When the protocol is complete, remove the Agilent Deep Well plate containing the AMPure XP bead suspension from position 5 of the Bravo deck.

11 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 **02 Cleanup_LibPrep_XT_HS2_ILM** protocol (refer to **Table 35** on page 61). Use the plate within the next 24 hours.

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 Purification

The pre-capture PCR cleanup protocols (**04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM** and **04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM**) require a bead plate containing 50 µL of beads in each well. Use the **AMPureXP_Aliquot_PreCap** protocol to prepare the bead plate needed for purification of pre-capture PCR products.

Prepare the NGS Bravo and reagents for the AMPureXP_Aliquot_PreCap protocol

1 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.

2 Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.

   a Verify that the AMPure XP bead 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 pre-capture PCR cleanup protocol (each column accommodates 8 amplified DNA samples).

Load the NGS Bravo

3 Load the Bravo deck according to **Table 14**.

Table 14 Initial Bravo deck configuration for AMPureXP_Aliquot_PreCap protocol

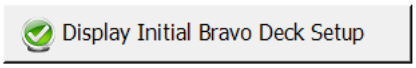
Location	Content
2	New tip box
5	Empty Agilent Deep Well Plate
6	Reservoir of AMPure XP bead suspension prepared in **step 2**
8	Empty tip box

Run VWorks protocol AMPureXP_Aliquot_PreCap

4 On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot_PreCap** protocol.

5 Select the number of columns of samples to be processed.

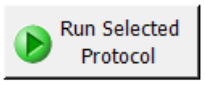
6 Click **Display Initial Bravo Deck Setup**.



7 Verify that the Bravo deck has been set up as displayed on the right side of the form.

8 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **AMPureXP_Aliquot_PreCap** protocol takes approximately 5 minutes. The protocol directs the NGS Bravo to aliquot 50 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent Deep Well plate.

10 When the protocol is complete, remove the Agilent Deep Well plate containing the AMPure XP bead suspension from position 5 of the Bravo deck.

11 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 **04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM** or **04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM** protocol (refer to **Table 42** on page 70). Use the plate within the next 24 hours.

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 **06c PoolingConcentration_XT_HS2_ILM** protocol is part of the pre-capture pooling workflow. It requires a bead plate containing 180 µL of beads in each well. Use the **AMPureXP_Aliquot_Pooling** protocol to prepare the bead plate needed for concentrating the DNA library pools.

The bead plate for the **06c PoolingConcentration_XT_HS2_ILM** 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 42.

Prepare the NGS Bravo and reagents for the AMPureXP_Aliquot_Pooling protocol

1 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.

2 Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.

   a Verify that the AMPure XP bead 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 **06c PoolingConcentration_XT_HS2_ILM** protocol (each column accommodates 8 DNA library pools).

Load the NGS Bravo

3 Load the Bravo deck according to **Table 15**.

Table 15 Initial Bravo deck configuration for AMPureXP_Aliquot_Pooling protocol

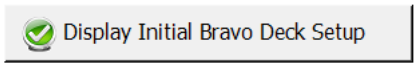
Location	Content
2	New tip box
5	Empty Agilent Deep Well Plate
6	Reservoir of AMPure XP bead suspension prepared in **step 2**
8	Empty tip box

Run VWorks protocol AMPureXP_Aliquot_Pooling

4 On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot_Pooling** protocol.

5 Select the number of columns of samples to be processed.

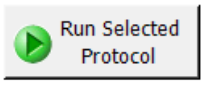
6 Click **Display Initial Bravo Deck Setup**.



7 Verify that the Bravo deck has been set up as displayed on the right side of the form.

8 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **AMPureXP_Aliquot_Pooling** protocol takes approximately 5 minutes. The protocol directs the NGS Bravo to aliquot 180 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent Deep Well plate.

10 When the protocol is complete, remove the Agilent Deep Well plate containing the AMPure XP bead suspension from position 5 of the Bravo deck.

11 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 **06c PoolingConcentration_XT_HS2_ILM** protocol (refer to **Table 50** on page 91). Use the plate within the next 24 hours.

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 **11 Cleanup_Post-CapPCR__XT_HS2_ILM** protocol requires a bead plate containing 50 µL of beads in each well. Use the **AMPureXP_Aliquot_PostCap** protocol to prepare the bead plate needed for purification of post-capture PCR products.

Prepare the NGS Bravo and reagents for the AMPureXP_Aliquot_PostCap protocol

1 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.

2 Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.

- **a** Verify that the AMPure XP bead 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 **11 Cleanup_Post-CapPCR__XT_HS2_ILM** protocol (each column accommodates 8 indexed libraries).

Load the NGS Bravo

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

Table 16 Initial Bravo deck configuration for AMPureXP_Aliquot_PostCap protocol

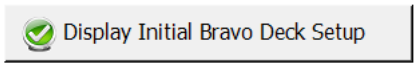
Location	Content
2	New tip box
5	Empty Agilent Deep Well Plate
6	Reservoir of AMPure XP bead suspension prepared in **step 2**
8	Empty tip box

Run VWorks protocol AMPureXP_Aliquot_PostCap

4 On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot_PostCap** protocol.

5 Select the number of columns of samples to be processed.

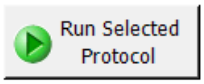
6 Click **Display Initial Bravo Deck Setup**.



7 Verify that the Bravo deck has been set up as displayed on the right side of the form.

8 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **AMPureXP_Aliquot_PostCap** protocol takes approximately 5 minutes. The protocol directs the NGS Bravo to aliquot 50 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent Deep Well plate.

10 When the protocol is complete, remove the Agilent Deep Well plate containing the AMPure XP bead suspension from position 5 of the Bravo deck.

11 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 **11 Cleanup_Post-CapPCR_XT_HS2_ILM** protocol (refer to **Table 74** on page 117). Use the plate within the next 24 hours.

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

4 Sample Preparation

For an overview of the SureSelect XT HS2 target enrichment workflow, see **Figure 2** on page 29. This section contains instructions for automated gDNA library preparation for the Illumina paired-read sequencing platform. For each sample to be sequenced, an individual indexed and molecular-barcoded library is prepared.

The library preparation protocol is compatible with both high-quality gDNA prepared from fresh or fresh frozen samples and lower-quality DNA prepared from FFPE samples. Modifications required for FFPE samples are included throughout the protocol steps. For a summary of modifications for FFPE samples see **Chapter 7** on **page 137**.

The protocol requires 10 ng to 200 ng of input DNA, with adjustments to DNA input amount or quantification method required for some FFPE samples. For optimal 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.

Step 1. Prepare and analyze quality of genomic DNA samples

Preparation of high-quality gDNA from fresh biological samples

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

NOTE

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

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

Additional qualification of DNA samples is not required for DNA derived from fresh biological samples. Depending on the type of sample fragmentation method to be used, proceed to either **"Method 1: Prepare fragmented DNA by enzymatic fragmentation"** on page 48 or **"Method 2. Prepare fragmented DNA by mechanical shearing"** on page 54.

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.

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

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

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 17**.

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 17 SureSelect XT HS2 DNA input modifications based on ΔΔCq DNA integrity score

Protocol Parameter	non-FFPE Samples	FFPE Samples	
		ΔΔCq≤1*	ΔΔCq >1
DNA input for Library Preparation	10 ng to 200 ng DNA, based on Qubit Assay	10 ng to 200 ng DNA, based on Qubit Assay	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 18** to determine the recommended amount of input DNA for the sample.

Table 18 SureSelect XT HS2 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 38** on page 64. Samples with differing PCR cycle number requirements must be processed in separate library preparation runs.

Step 2. Fragment the DNA

The SureSelect XT HS2 target enrichment workflow supports two different methods for preparing fragmented DNA.

- Method 1 uses enzymatic fragmentation, with liquid-handling steps executed by automation protocols. The instructions are described in **"Method 1: Prepare fragmented DNA by enzymatic fragmentation"** on page 48.
- Method 2 uses mechanical shearing, and is described in **"Method 2. Prepare fragmented DNA by mechanical shearing"** on page 54.

Select the desired option and then follow the instructions in the appropriate section.

Method 1: Prepare fragmented DNA by enzymatic fragmentation

In method 1, the NGS Bravo completes the liquid-handling steps for enzymatic fragmentation of the DNA samples using protocol **00a EnzFrag_XT_HS2_ILM**. After the Bravo completes the liquid-handling steps, you transfer the PCR plate to a thermal cycler for incubation. You then transfer the PCR plate back to the Bravo deck to run a dilution protocol called **00b EnzFrag_Dil_XT_HS2_ILM** that dilutes the samples to 50-µL volumes.

Method 1 uses the components listed in **Table 19**. Thaw and mix each component as directed in **Table 19** before use. Before starting the run, you need to prepare the Fragmentation master mix (with overage) without the DNA sample. **Table 22** provides Fragmentation master mix volumes based on the number of columns of samples in the run.

Table 19 Reagents thawed before use in protocol

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
5X SureSelect Fragmentation Buffer (blue cap)	SureSelect Enzymatic Fragmentation Kit, –20°C	Thaw on ice then keep on ice	Vortexing	**page 50**
SureSelect Fragmentation Enzyme (green cap)	SureSelect Enzymatic Fragmentation Kit, –20°C	Place on ice just before use	Inversion	**page 50**

Prepare the NGS Bravo for protocol 00a EnzFrag_XT_HS2_ILM

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. 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.
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"** on page 21. 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.

Pre-program the thermal cycler for the fragmentation reaction

5 Pre-program a thermal cycler using the program in **Table 20**. Start the program, then immediately pause the program.

Table 20 Thermal cycler program for enzymatic fragmentation*

Step	Temperature	Time
Step 1	37°C	Varies -- see **Table 21**
Step 2	65°C	5 minutes
Step 3	4°C	Hold

* Use a reaction volume setting of 20 µL, if required for thermal cycler set up.

Optimal fragmentation conditions may vary based on the NGS read length to be used in the workflow. Refer to **Table 21** below for the duration at 37°C appropriate for your sample type and required NGS read length.

Table 21 Fragmentation duration based on sample type and NGS read length

NGS read length requirement	Target fragment size	Duration of 37°C incubation step	
		High-quality DNA samples	FFPE DNA samples
2 × 100 reads	150 to 200 bp	25 minutes	25 minutes
2 × 150 reads	180 to 250 bp	15 minutes	25 minutes

Prepare the sample plate for fragmentation

6 In the wells of the PCR plate, dilute 10–200 ng of each gDNA sample with nuclease-free water to a final volume of 15 µL. Use the PCR plate that is to be placed in the thermal cycler for the enzymatic fragmentation program.

See **Table 17** or **Table 18** for FFPE DNA input guidelines based on the measured DNA quality in each sample.

Prepare the Fragmentation master mix

7 Prepare the appropriate volume of Fragmentation master mix by combining the reagents in **Table 22**. Mix well by pipetting up and down 20 times or seal the tube and vortex at high speed for 5–10 seconds. Spin briefly to remove any bubbles and keep on ice.

Table 22 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 22 Preparation of Fragmentation master mix for 1 to 12 columns of samples

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	2 µL	42.5	59.5	76.5	97.8	119.0	136.0	153.0	170.0	191.3	208.3	225.3	253.8
5X SureSelect Fragmentation Buffer (blue cap)	2 µL	42.5	59.5	76.5	97.8	119.0	136.0	153.0	170.0	191.3	208.3	225.3	253.8
SureSelect Fragmentation Enzyme (green cap)	1 µL	21.3	29.8	38.3	48.9	59.5	68.0	76.5	85.0	95.6	104.1	112.6	126.9
Total Volume	**5 µL**	**106.3**	**148.8**	**191.3**	**244.5**	**297.5**	**340.0**	**382.5**	**425.0**	**478.2**	**620.7**	**583.2**	**634.4**

Prepare the master mix source plate (Eppendorf twin.tec or Armadillo plate)

8 Prepare the master mix source plate for the run as indicated in **Table 23**. Add the indicated volume of master mix to all wells of the indicated column 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 3**.

Use either an **Eppendorf twin.tec** plate or an **Armadillo** plate as the master mix source plate, as indicated in the Processing Plate setting on the form.

Table 23 Preparation of the master mix source plate for 00a EnzFrag_XT_HS2_ILM protocol

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Selected Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Fragmentation master mix	Column 1 (A1-H1)	12.5	17.5	22.5	28.8	35.0	40.0	45.8	51.5	57.3	63.0	68.8	75.0

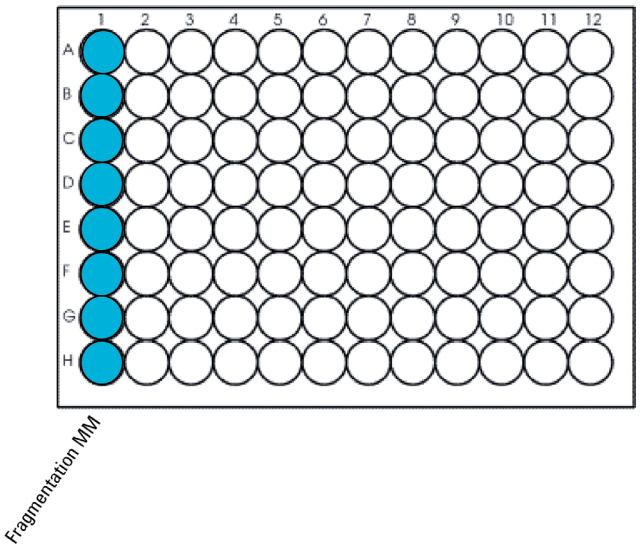


Figure 3 Configuration of the master mix source plate for protocol **00a EnzFrag_XT_HS2_ILM**

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

10 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 Bravo

11 Load the Bravo deck according to **Table 24**.

Table 24 Initial Bravo deck configuration for 00a EnzFrag_XT_HS2_ILM protocol

Location	Content
2	New tip box
4	gDNA samples in PCR plate seated in red insert (PCR plate type must be specified on setup form under Parameter 2)
5	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
8	Empty tip box
9	Fragmentation master mix source plate, unsealed

Run VWorks protocol 00a EnzFrag_XT_HS2_ILM

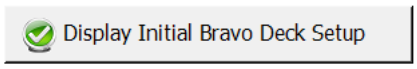
12 On the SureSelect setup form, under **Select protocol to execute**, select the **00a EnzFrag_XT_HS2_ILM** protocol.

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

14 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

15 Select the number of columns of samples to be processed.

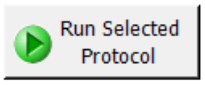
16 Click **Display Initial Bravo Deck Setup**.



17 Verify that the Bravo deck has been set up as displayed on the right side of the form.

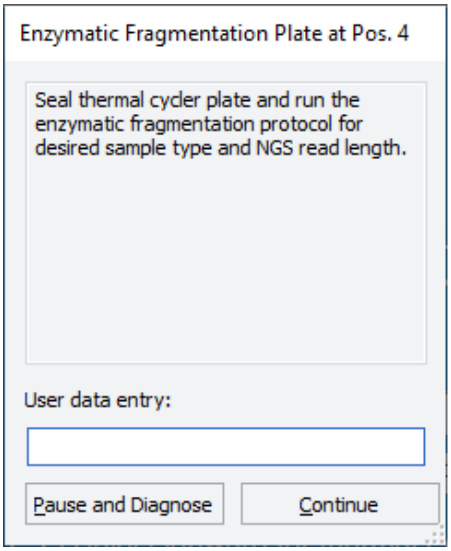
18 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **00a EnzFrag_XT_HS2_ILM** protocol takes approximately 10 minutes. Once complete, the samples are ready for fragmentation (performed in the pre-programmed thermal cycler). The samples are located in the plate at position 4 of the Bravo deck.

20 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.



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

22 Immediately place the sample plate in the thermal block. Close the lid and resume the thermal cycler program in **Table 20**.

23 From the Bravo deck, remove the Eppendorf twin.tec or Armadillo plate that was used as the Fragmentation master mix source plate from position 9 and set it aside. You will use this same plate again for the **03 Pre-CapPCR_XT_HS2_ILM** protocol as described in **"Prepare the pre-capture PCR master mix and master mix source plate"** on page 65.

Prepare the NGS Bravo and reagents for protocol 00b EnzFrag_Dil_XT_HS2_ILM

24 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.

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

26 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, you may retain the Agilent shallow well reservoir for use in the **02 Cleanup_LibPrep_XT_HS2_ILM** protocol (see **"Step 4. Purify adaptor-ligated DNA using AMPure XP beads"** on page 61) if you are running that protocol today.

Load the NGS Bravo

27 Load the Bravo deck according to **Table 25**.

Table 25 Initial Bravo deck configuration for 00b EnzFrag_Dil_XT_HS2_ILM protocol

Location	Content
2	New tip box
4	PCR plate containing fragmented DNA samples seated in red insert (PCR plate type must be specified on setup form under Parameter 2)
5	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
6	Nuclease-free water reservoir from **step 26**
8	Empty tip box

Run VWorks protocol 00b EnzFrag_Dil_XT_HS2_ILM

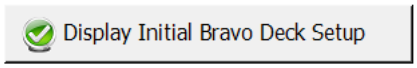
28 On the SureSelect setup form, under **Select protocol to execute**, select the **00b EnzFrag_Dil_XT_HS2_ILM** protocol.

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

30 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

31 Select the number of columns of samples to be processed.

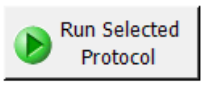
32 Click **Display Initial Bravo Deck Setup**.



33 Verify that the Bravo deck has been set up as displayed on the right side of the form.

34 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **00b EnzFrag_Dil_XT_HS2_ILM** protocol takes approximately 5 minutes. Once complete, the samples are ready for library preparation. The samples are located in the processing plate at position 1 of the Bravo deck.

NOTE

This is not a stopping point in the workflow, and analysis of the enzymatically-fragmented samples is not required prior to library preparation. Moreover, electrophoretic analysis of the fragmented samples may produce misleading results due to the presence of agents that affect DNA fragment migration.

Proceed directly to **"Step 3. Prepare adaptor-ligated libraries"** on page 56.

Method 2. Prepare fragmented DNA by mechanical shearing

In method 2, the 50-µL gDNA samples are mechanically sheared using conditions optimized for either high-quality or FFPE DNA.

The target fragment size and corresponding shearing conditions may vary for workflows using different NGS read lengths. See **Table 26** for guidelines. Complete shearing instructions are provided on **page 55**.

Table 26 Covaris shearing duration based on NGS length

NGS read length requirement	Target fragment size	Shearing duration for high-quality DNA samples	Shearing duration for FFPE DNA samples
20 × 100 reads	150 to 200 bp	2 × 120 seconds	240 seconds
20 × 150 reads	180 to 250 bp	2 × 60 seconds	240 seconds

NOTE

Shearing protocols have been optimized using a Covaris model E220 instrument and the Covaris 96 microTUBE plate. Consult the manufacturer's recommendations for use of other Covaris instruments or sample holders to achieve the desired target DNA fragment size.

1 Set up the Covaris E220 instrument. Refer to the Covaris instrument user guide for details.
   - **a** Check that the water in the Covaris tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate in use.
   - **b** Check that the water covers the visible glass part of the tube.
   - **c** On the instrument control panel, push the Degas button. Degas the instrument according to the manufacturer's recommendations, typically 30–60 minutes.
   - **d** Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C. Consult the manufacturer's recommendations for addition of coolant fluids to prevent freezing.
2 Prepare the DNA samples for the run by diluting 10–200 ng of each gDNA sample with 1X Low TE Buffer (10 mM Tris- HCl, pH 7.5–8.0, 0.1 mM EDTA) to a final volume of 50 µL. Vortex well to mix, then spin briefly to collect the liquid. Keep the samples on ice.

   See **Table 17** or **Table 18** for FFPE DNA input guidelines based on the measured DNA quality in each sample.

NOTE

Do not dilute samples to be sheared using water. Shearing samples in water reduces the overall library preparation yield and complexity.

3 Vortex each sample dilution to mix, then spin briefly to collect the liquid. Keep the samples on ice.

4 Complete the DNA shearing steps below for each of the gDNA samples.

Transfer the 50-µL DNA sample into a Covaris 96 microTUBE Plate, using a tapered pipette tip to slowly transfer the sample through the pre-split foil seal. Seal the plate with the provided foil seal.

a Spin the 96 microTUBE Plate for 30 seconds to collect the liquid and to remove any bubbles from the bottom of the tube.

b Load the 96 microTUBE Plate onto the loading tray and shear the DNA with the settings in **Table 27**.

Table 27 Shear settings for Covaris E-series instrument (SonoLab software v7 or later)

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

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

- Shear for 120 seconds
- Spin the 96 microTUBE Plate for 10 seconds
- Shear for additional 120 seconds
- Spin the 96 microTUBE Plate for 10 seconds

c Insert a pipette tip through the foil seal, then slowly remove the sheared DNA.

d Transfer the sheared DNA sample (approximately 50 µL) to a sample well of a 96-well processing plate (Eppendorf twin.tec or Armadillo plate). Keep the samples on ice.

e After transferring the DNA sample, spin the 96 microTUBE Plate briefly to collect any residual sample volume. Transfer any additional collected liquid to the sample well used in **step d**.

NOTE

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

Step 3. Prepare adaptor-ligated libraries

This step uses automation protocol **01 LibraryPrep_XT_HS2_ILM**.

In this step, the NGS Bravo completes the DNA end modification steps required for SureSelect target enrichment, including end-repair, dA-tailing, and ligation of the molecular-barcoded adaptor.

This step uses the components listed in **Table 28**. Thaw and mix each component as directed in **Table 28** before use. Before starting the run, you need to prepare master mixes (with overage) for each step, without the DNA sample. Master mixes for runs that include 1, 2, 3, 4, 6, and 12 columns (including overage) are shown in reagent preparation tables.

Table 28 Reagents thawed before use in protocol

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
End Repair-A Tailing Buffer (bottle)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	**page 57**
Ligation Buffer (bottle)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	**page 58**
End Repair-A Tailing Enzyme Mix (orange cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Place on ice just before use	Inversion	**page 57**
T4 DNA Ligase (blue cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Place on ice just before use	Inversion	**page 58**
SureSelect XT HS2 Adaptor Oligo Mix (white cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	**page 58**

Prepare the NGS Bravo for protocol 01 LibraryPrep_XT_HS2_ILM

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Pre-set the temperature of Bravo deck position 4 to 79°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.
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. 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.

Prepare the DNA End-Repair/dA-Tailing master mix

5 Prepare the appropriate volume of End Repair/dA-Tailing master mix, using volumes listed in **Table 29** and using the liquid handling steps specified below.

Table 29 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

a Vortex the thawed vial of End Repair-A Tailing Buffer for 15 seconds at high speed to ensure homogeneity. Visually inspect the solution; if any solids are observed, continue vortexing until all solids are dissolved.

CAUTION The End Repair-A Tailing Buffer used in this step must be mixed thoroughly by vortexing at high speed for 15 seconds before removing an aliquot for use. When combining with other reagents, mix well by pipetting up and down 15–20 times using a pipette set to at least 80% of the mixture volume.

b Slowly pipette the End Repair-A Tailing Buffer into a 1.5-mL Eppendorf tube or conical vial, ensuring that the full volume is dispensed.

c Slowly add the End Repair-A Tailing Enzyme Mix, rinsing the enzyme tip with buffer solution after addition. Mix well by pipetting up and down 15–20 times. (For large volumes, instead vortex at medium speed for 25–30 seconds to ensure homogeneity.) Spin briefly to collect the liquid and keep on ice.

Table 29 Preparation of End Repair/dA-Tailing master mix

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
End Repair-A Tailing Buffer (yellow cap or bottle)	16 µL	204.0	340.0	476.0	612.0	748.0	884.0	1042.7	1201.3	1360.0	1518.7	1677.3	1836.0
End Repair-A Tailing Enzyme Mix (orange cap)	4 µL	51.0	85.0	119.0	153.0	187.0	221.0	260.7	300.3	340.0	379.7	419.3	459.0
Total Volume	**20 µL**	**255.0**	**425.0**	**595.0**	**765.0**	**935.0**	**1105.0**	**1303.4**	**1501.6**	**1700.0**	**1898.4**	**2096.6**	**2295.0**

Prepare the Ligation master mix

6 Prepare the appropriate volume of Ligation master mix, using volumes listed in **Table 30** and using the liquid handling steps specified below.

Table 30 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

a Vortex the thawed vial of Ligation Buffer for 15 seconds at high speed to ensure homogeneity.

CAUTION The Ligation Buffer used in this step is viscous. Mix thoroughly by vortexing at high speed for 15 seconds before removing an aliquot for use. When combining with other reagents, mix well by pipetting up and down 15–20 times using a pipette set to at least 80% of the mixture volume.

b Slowly pipette the Ligation Buffer into a 1.5-mL Eppendorf tube or conical vial, ensuring that the full volume is dispensed.

c Slowly add the T4 DNA Ligase, rinsing the enzyme tip with buffer solution after addition. Mix well by slowly pipetting up and down 15–20 times. (For large volumes, instead vortex at medium speed for 25–30 seconds to ensure homogeneity.) Spin briefly to collect the liquid.

Table 30 Preparation of Ligation master mix

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Ligation Buffer (purple cap or bottle)	23 µL	293.3	488.8	684.3	879.8	1075.3	1270.8	1515.1	1759.5	2003.9	2248.3	2492.6	2737.0
T4 DNA Ligase (blue cap)	2 µL	25.5	42.5	59.5	76.5	93.5	110.5	127.5	153.0	174.3	195.5	216.8	238.0
Total Volume	**25 µL**	**318.8**	**531.3**	**743.8**	**956.3**	**1168.8**	**1381.3**	**1642.6**	**1912.5**	**2178.2**	**2443.8**	**2709.4**	**2975.0**

Prepare the Adaptor Oligo Mix

7 Prepare the appropriate volume of Adaptor Oligo Mix dilution, according to **Table 31**. Mix well using a vortex mixer and keep on ice.

Table 31 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 31 Preparation of Adaptor Oligo Mix dilution

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	2.5 µL	42.5	63.8	85.0	106.3	127.5	148.8	170.0	191.3	212.5	233.8	255.0	276.3
SureSelect XT HS2 Adaptor Oligo Mix (white cap)	5 µL	85.0	127.5	170.0	212.5	255.0	297.5	340.0	382.5	425.0	467.5	510.0	552.5
Total Volume	**7.5 µL**	**127.5**	**191.3**	**255.0**	**318.8**	**382.5**	**446.3**	**510.0**	**573.8**	**637.5**	**701.3**	**765.0**	**828.8**

Prepare the master mix source plate

8 Prepare the **Agilent Deep Well** master mix source plate containing the mixtures prepared in **step 5** through **step 7**. Add the volumes indicated in **Table 32** of each mixture to all wells of the indicated column of the Agilent Deep Well plate. Keep the master mixes on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 4**.

Table 32 Preparation of the master mix source plate for 01 LibraryPrep_XT_HS2_ILM protocol

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Agilent Deep Well Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
End Repair-dA Tailing master mix	Column 1 (A1-H1)	31.0	52.0	73.0	94.0	115.0	136.0	158.8	182.0	205.8	230.0	254.8	280.0
Ligation master mix	Column 2 (A2-H2)	36.0	62.0	88.0	114.0	140.0	166.0	195.7	226.5	258.3	291.1	325.1	360.0
Adaptor Oligo Mix dilution	Column 3 (A3-H3)	15.0	22.5	30.0	37.5	45.0	52.5	60.6	68.8	76.9	85.0	93.1	101.3

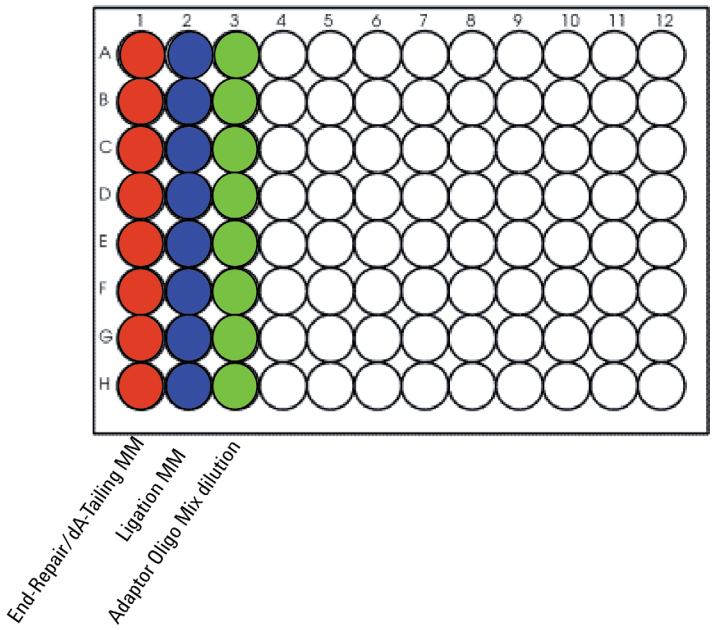


Figure 4 Configuration of the **Agilent Deep Well** master mix source plate for protocol **01 LibraryPrep_XT_HS2_ILM**

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

10 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 Bravo

11 Load the Bravo deck according to **Table 33**.

Table 33 Initial Bravo deck configuration for 01 LibraryPrep_XT_HS2_ILM protocol

Location	Content
1	Processing plate (Eppendorf twin.tec or Armadillo plate) containing sheared gDNA samples
2	New tip box
3	Empty processing plate (Eppendorf twin.tec or Armadillo plate) -- for aliquots of Adaptor Oligo Mix
4	Empty red insert
5	Empty processing plate (Eppendorf twin.tec or Armadillo plate) -- for aliquots of End Repair-dA Tailing master mix
7	Empty processing plate (Eppendorf twin.tec or Armadillo plate) -- for aliquots of Ligation master mix
8	Empty tip box
9	Library Prep master mix source plate, unsealed

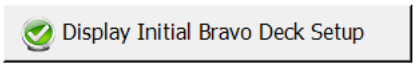
Run VWorks protocol 01 LibraryPrep_XT_HS2_ILM

12 On the SureSelect setup form, under **Select protocol to execute**, select the **01 LibraryPrep_XT_HS2_ILM** protocol.

13 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

14 Select the number of columns of samples to be processed.

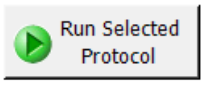
15 Click **Display Initial Bravo Deck Setup**.



16 Verify that the Bravo deck has been set up as displayed on the right side of the form.

17 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **01 LibraryPrep_XT_HS2_ILM** protocol takes approximately 1.5 hours. Once complete, the adaptor-ligated DNA samples are located in the selected processing plate at position 9 of the Bravo deck.

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

Step 4. Purify adaptor-ligated DNA using AMPure XP beads

This step uses automation protocol **02 Cleanup_LibPrep_XT_HS2_ILM**.

In this step the NGS Bravo combines the adaptor-ligated samples with AMPure XP beads and then collects and washes the bead-bound DNA.

Prepare the NGS Bravo and reagents for protocol 02 Cleanup_LibPrep_XT_HS2_ILM

1 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.

2 Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in **Table 34**. See **page 19** for more information on how to do this step.

Table 34 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
4	45°C	Inheco Multi TEC control touchscreen (CPAC 2-1)
6	4°C	Inheco Multi TEC control touchscreen (CPAC 2-2)

3 Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water. If available, use the same Agilent shallow well reservoir that was used in the **00b EnzFrag_Dil_XT_HS2_ILM** protocol.

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, you may retain the Agilent shallow well reservoir for use in the selected Cleanup_Pre-CapPCR protocol (see **"Step 6. Purify amplified DNA using AMPure XP beads"** on page 69) if you are running that protocol today.

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

Load the NGS Bravo

5 Load the Bravo deck according to **Table 35**.

Table 35 Initial Bravo deck configuration for 02 Cleanup_LibPrep_XT_HS2_ILM

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
5	Aliquotted AMPure XP beads in Agilent Deep Well plate from **page 38** (80 µL of beads/well)
6	Adaptor-tagged DNA samples in processing plate (Eppendorf twin.tec or Armadillo plate)
8	Empty tip box
9	70% ethanol reservoir from **step 4**

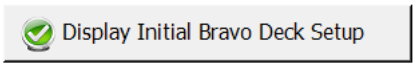
Run VWorks protocol 02 Cleanup_LibPrep_XT_HS2_ILM

6 On the SureSelect setup form, under **Select Protocol**, select the **02 Cleanup_LibPrep_XT_HS2_ILM** protocol.

7 Select the number of columns of samples to be processed.

8 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

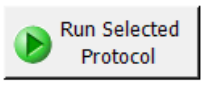
9 Click **Display Initial Bravo Deck Setup**.



10 Verify that the Bravo deck has been set up as displayed on the right side of the form.

11 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **02 Cleanup_LibPrep_XT_HS2_ILM** protocol takes approximately 40 minutes. An operator must be present during the run to complete tip box replacement and to transfer the water reservoir to the Bravo deck, when directed by the VWorks prompt shown below.

Once the protocol is complete, the purified DNA samples are located in the Agilent Deep Well plate at position 7 of the Bravo deck.

Step 5. Amplify adaptor-ligated libraries

This step uses automation protocol **03 Pre-CapPCR_XT_HS2_ILM**.

In this step, the NGS Bravo completes the liquid handling steps for amplification and dual-indexing of the adaptor-ligated DNA samples. After the NGS Bravo completes the liquid-handling steps, you transfer the PCR plate to a thermal cycler for amplification.

This step uses the components listed in **Table 36**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 36 Reagents for pre-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Pipette up and down 15–20 times	**page 65**
5× Herculase II Reaction Buffer (clear cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	**page 65**
SureSelect XT HS2 Index Primer Pairs	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR),* –20°C	Vortexing	**page 65**

* Indexing primer pairs are provided in a 96-well plate.

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 XT HS2 Index Primer Pairs 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 NGS Bravo for protocol 03 Pre-CapPCR_XT_HS2_ILM

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. 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.
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.

Pre-program the thermal cycler for pre-capture PCR

4 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 37**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the automation run.

Table 37 Pre-Capture PCR Thermal Cycler Program*

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8 to 14, based on input DNA quality and quantity (see **Table 38**)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

* When setting up the thermal cycler program, use a reaction volume setting of 50 µL.

Table 38 Pre-capture PCR cycle number recommendations

Quality of Input DNA	Quantity of Input DNA	Cycles
Intact DNA from fresh sample	100 to 200 ng	8 cycles
	50 ng	9 cycles
	10 ng	11 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 SureSelect XT HS2 Index Primer Pairs

5 Using a multichannel pipette, transfer 5 µL of each SureSelect XT HS2 Index Primer Pair from the 96-well plate in which the primer pairs 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 primer pairs is loaded onto the Bravo deck in **step 10** on **page 66** for the **03 Pre-CapPCR_XT_HS2_ILM** protocol.

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

6 Prepare the appropriate volume of pre-capture PCR master mix, according to **Table 39**. Vortex at medium speed for 15–20 seconds and keep on ice.

Table 39 Preparation of Pre-Capture PCR Master Mix

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
5× Herculase II Buffer with dNTPs (clear cap)	10 µL	170	255	340	425	510.0	574	656.0	738.0	820.0	902.0	984.0	1066
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17	25.5	34	42.5	51.0	57.4	65.6	73.8	82.0	90.2	98.4	106.6
Total Volume	**11 µL**	**187**	**280.5**	**374**	**467.5**	**561.0**	**631.4**	**721.6**	**811.8**	**902.0**	**992.2**	**1082.4**	**1172.6**

7 Using the same master mix source plate (Eppendorf twin.tec or Armadillo plate) that was used for the Fragmentation master mix in the **00a EnzFrag_XT_HS2_ILM** protocol, add the volume of PCR master mix indicated in **Table 40** to all wells of column 2 of the master mix source plate. If you did not use the enzymatic fragmentation method to fragment the DNA, then add the PCR master mix to column 2 of a fresh processing plate (either an Eppendorf twin.tec or an Armadillo plate, as indicated in the Processing Plate setting on the form). The final configuration of the master mix source plate is shown in **Figure 5**.

Table 40 Preparation of the master mix source plate for 03 Pre-CapPCR_XT_HS2_ILM protocol

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Selected Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Pre-Capture PCR Master Mix	Column 2 (A2-H2)	22	33	44	55	66	77	88	99	110	121	132	143

CAUTION Make sure to add the Pre-Capture PCR master mix to column 2 of the source plate.

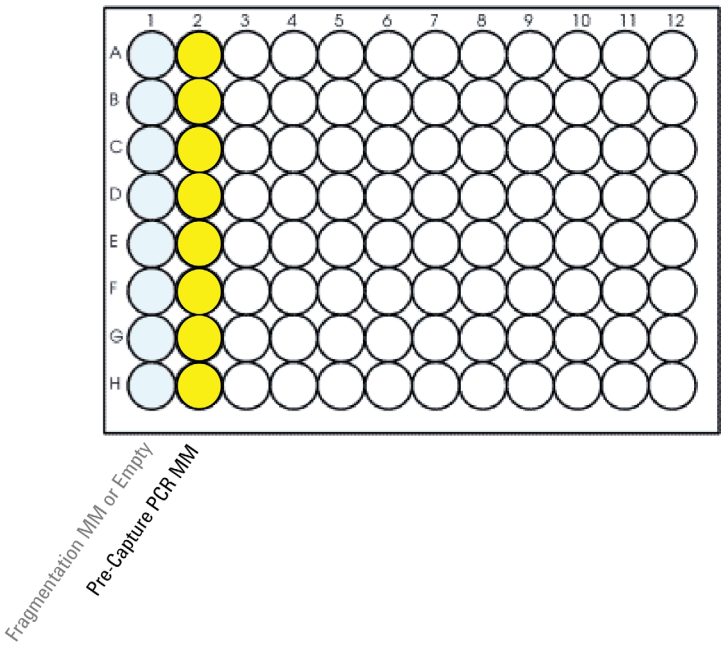


Figure 5 Configuration of the master mix source plate for protocol **03 Pre-CapPCR_XT_HS2_ILM**. The master mix dispensed during a previous protocol is shown in light shading.

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

9 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 Bravo

10 Load the Bravo deck according to **Table 41**.

Table 41 Initial Bravo deck configuration for 03 Pre-CapPCR_XT_HS2_ILM protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
6	SureSelect XT HS2 Index Primer Pairs for ILM in PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
7	Adaptor-ligated DNA samples in Agilent Deep Well plate
8	Empty tip box
9	Master mix source plate (Eppendorf twin.tec or Armadillo plate) containing PCR master mix in column 2 (unsealed)

Run VWorks protocol 03 Pre-CapPCR_XT_HS2_ILM

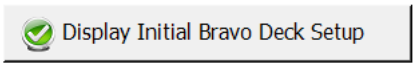
11 On the SureSelect setup form, under **Select protocol to execute**, select the **03 Pre-CapPCR_XT_HS2_ILM** protocol.

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

13 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

14 Select the number of columns of samples to be processed.

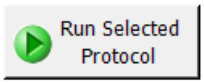
15 Click **Display Initial Bravo Deck Setup**.



16 Verify that the Bravo deck has been set up as displayed on the right side of the form.

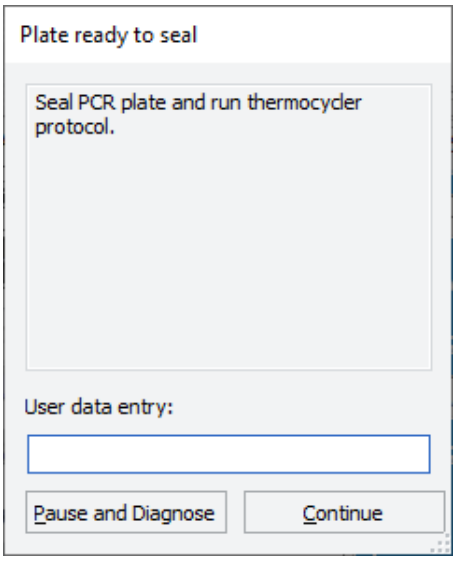
17 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **03 Pre-CapPCR_XT_HS2_ILM** protocol takes approximately 10 minutes. Once complete, the PCR-ready samples, containing prepped DNA and PCR master mix, are located in the PCR plate at position 6 of the Bravo deck.

19 When you see the following prompt, remove the PCR plate from position 6 of the Bravo deck and seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 seconds.



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

21 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 37**. Once the cycler has reached 98°C, immediately place the sample plate in the thermal block and close the lid.

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

Retain the master mix source plate (Eppendorf twin.tec or Armadillo plate) containing the Pre-Capture PCR master mix located at position 9 of the Bravo deck for later use in the **05 TS_D1000** protocol (see **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape"** on page 72).

Step 6. Purify amplified DNA using AMPure XP beads

This step uses either automation protocol **04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM** or **04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM**.

In this step, the NGS Bravo transfers amplified DNA to an Agilent Deep Well plate containing AMPure XP beads, and then collects and washes the bead-bound DNA.

This step uses the aliquoted plate of AMPure XP beads that was prepared on **page 38**.

Prepare the NGS Bravo and reagents for the Cleanup_Pre-CapPCR protocol

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Pre-set the temperature of Bravo deck position 4 to 45°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.
3. 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.
4. Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water. If available, use the same Agilent shallow well reservoir that was used in the **02 Cleanup_LibPrep_XT_HS2_ILM** protocol.

   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, you may retain the water reservoir for use in either the **06a Aliquot_Libraries** or **06b Aliquot_Water** protocol (see **"Step 1. Prepare DNA for Hybridization"** on page 80) if you are running one of those protocols today.
5. Prepare an Agilent Deep Well reservoir containing 50 mL of freshly-prepared 70% ethanol.

Load the NGS Bravo

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

Table 42 Initial Bravo deck configuration for the 04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM or 04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
3	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
5	Aliquotted AMPure XP beads in Agilent Deep Well plate from **page 38** (50 µL of beads/well)
6	Amplified DNA libraries in unsealed PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
8	Empty tip box
9	70% ethanol reservoir from **step 5**

Run the appropriate Cleanup_Pre-CapPCR protocol

7 On the SureSelect setup form, under **Select protocol to execute**, select one of the Cleanup_Pre-CapPCR 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 **04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM**.
- If you are using the pre-capture pooling workflow (i.e., pooling samples prior to hybridization with the Probe) then select the protocol **04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM**.

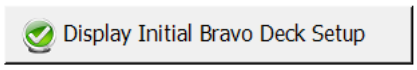
The 04a and 04b protocols use different elution volumes. Selecting the correct option is important for the downstream hybridization protocol.

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

9 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

10 Select the number of columns of samples to be processed.

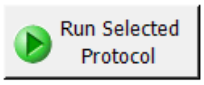
11 Click **Display Initial Bravo Deck Setup**.



12 Verify that the Bravo deck has been set up as displayed on the right side of the form.

13 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



The purification protocol takes approximately 40 minutes. An operator must be present during the run to complete tip box replacement and to transfer the water reservoir to the Bravo deck, when directed by the VWorks prompt shown below.

When complete, the purified DNA samples are in the selected processing plate located on Bravo deck position 3.

Step 7. Assess Library DNA quantity and quality

Sample analysis can be completed using one of two options.

- Option 1: Prepare the analytical assay plate using automation (protocol **05 TS_D1000**) and perform analysis on Agilent 4200 TapeStation. See **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape"** on page 72.
- Option 2: Prepare the analytical samples manually and perform analysis on Agilent 2100 Bioanalyzer, Agilent 4200 or 4150 TapeStation or Agilent 5200 Fragment Analyzer. See **"Option 2: Analysis using an equivalent platform (non-automated)"** on page 76.

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

This section describes use of automation protocol **05 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 Bravo and Sample Buffer source plate for protocol 05 TS_D1000

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Turn off the ThermoCube device (see **page 22**) to restore position 9 of the Bravo deck to room temperature.
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. Using the same processing source plate (Eppendorf twin.tec or Armadillo plate) that was used in the **03 Pre-CapPCR_XT_HS2_ILM** protocol, prepare the Sample Buffer source plate at room temperature. Add the volume of D1000 Sample Buffer indicated in **Table 43** to each well of column 3 of the plate.

   Make sure to add the D1000 Sample Buffer to column 3 of the source plate.

Table 43 Preparation of the Sample Buffer source plate for TS_D1000 protocol

Solution	Position on Source Plate	Volume (µL) of Sample Buffer added per Well of Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
D1000 Sample Buffer	Column 3 (A3-H3)	11.0	17.0	23.0	29.0	35.0	41.0	47.0	53.0	59.0	65.0	71.0	77.0

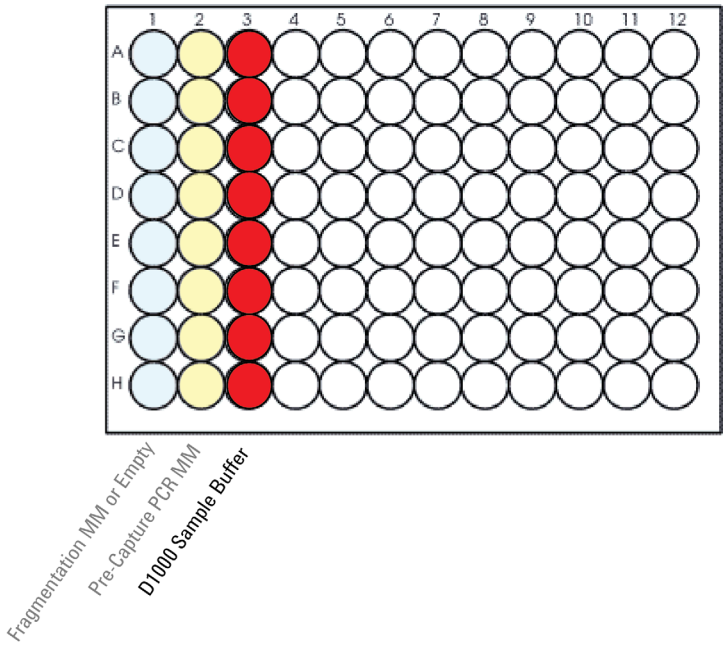


Figure 6 Configuration of the processing source plate for protocol **05 TS_D1000**. The master mixes dispensed during previous protocols are shown in light shading.

Load the NGS Bravo

5 Load the Bravo deck according to **Table 44**.

Table 44 Initial Bravo deck configuration for TS_D1000 protocol

Location	Content
2	New tip box
4	Amplified pre-capture libraries in Eppendorf twin.tec or Armadillo plate (unsealed)
6	Empty TapeStation analysis plate (Agilent p/n 5042-8502)
8	Empty tip box
9	Processing source plate (Eppendorf twin.tec or Armadillo plate) containing D1000 Sample Buffer in Column 3

CAUTION

To prevent damage to the Agilent 4200 TapeStation instrument and the Agilent NGS Bravo, use only the specified Agilent plates (Agilent p/n 5042-8502) for automated assay plate preparation.

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

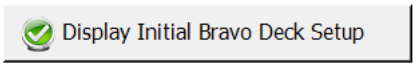
Run VWorks protocol 05 TS_D1000

6 On the SureSelect setup form, under **Select protocol to execute**, select **05 TS_D1000**.

7 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

8 Select the number of columns of samples to be processed.

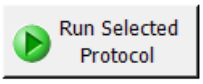
9 Click **Display Initial Bravo Deck Setup**.



10 Verify that the Bravo deck has been set up as displayed on the right side of the form.

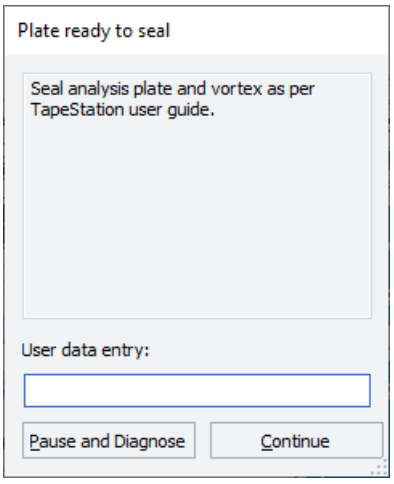
11 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **05 TS_D1000** protocol takes approximately 10 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 82**.

13 When prompted by VWorks as shown below, remove the TapeStation analysis 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

14 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.

15 Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 45** for guidelines). Sample electropherograms are shown in **Figure 7** (library prepared from high-quality DNA), **Figure 8** (library prepared from medium-quality FFPE DNA), and **Figure 9** (library prepared from low-quality FFPE DNA).

The appearance of an additional low molecular weight peak indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to that seen in the example electropherogram in **Figure 9**. See Troubleshooting information on **page 162** for additional considerations.

Table 45 Pre-capture library qualification guidelines

NGS read length for fragmentation protocol selection	Fragmentation method	Input DNA type	Expected library DNA fragment size peak position
2 × 100 reads	Mechanical shearing	Intact DNA	300 to 400 bp
		FFPE DNA	200 to 400 bp
	Enzymatic fragmentation	Intact DNA	300 to 400 bp
		FFPE DNA	200 to 400 bp
2 × 150 reads	Mechanical shearing	Intact DNA	330 to 450 bp
		FFPE DNA	200 to 450 bp
	Enzymatic fragmentation	Intact DNA	330 to 450 bp
		FFPE DNA	200 to 450 bp

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

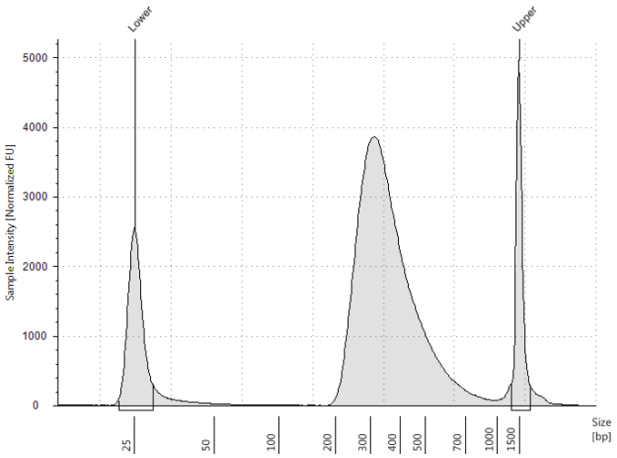


Figure 7 Pre-capture library prepared from a high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

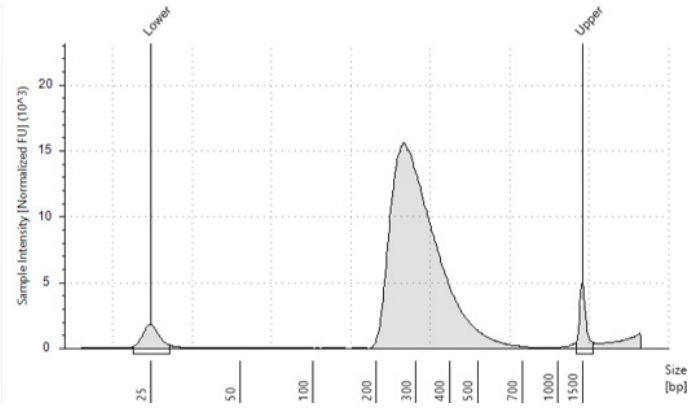


Figure 8 Pre-capture library prepared from a typical FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

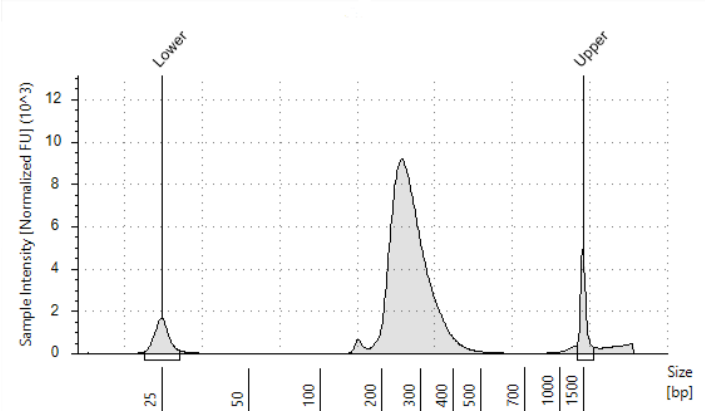


Figure 9 Pre-capture library prepared from a low-quality FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

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 Agilent 2100 BioAnalyzer, 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 7** through **Figure 9**). Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 45** for guidelines). **Table 46** includes links to assay instruction.

Table 46 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 sample mixed with 3 µL of D1000 sample buffer
Agilent 2100 BioAnalyzer system	DNA 1000 Kit	**Agilent DNA 1000 Kit Guide**	1 µL of sample
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 sample

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.

5 Hybridization

This chapter describes the steps to complete the hybridization and capture steps using a SureSelect or ClearSeq Probe.

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

Step 1. Prepare DNA for Hybridization

The first step is to prepare the DNA libraries for hybridization, and this step differs depending on the sample pooling strategy.

- If you are pooling samples after hybridization to the Probe, then follow the steps in **"Option 1: Single-plex hybridization"** on page 80.
- If you are pooling samples prior to hybridization to the Probe, then follow the steps in **"Option 2: Multi-plex hybridization"** on page 83.

Option 1: Single-plex hybridization

This step uses automation protocol **06a Aliquot_Libraries**.

Follow the steps in this section if you are using the post-capture pooling workflow. If you are using the pre-capture pooling workflow, see **"Option 2: Multi-plex hybridization"** on page 83.

For each sample library prepared, do one hybridization and capture.

The hybridization reaction requires 500–1000 ng of prepared DNA in a volume of 12 µL. Use the maximum amount of prepared DNA available within this range.

Calculate sample volumes needed for hybridization

1 Using the DNA concentration for each sample determined on **page 72** to **page 76**, calculate the volume of each sample to be used for hybridization using the formula below:

Volume (µL) = 1000 ng/concentration (ng/µL)

If the concentration of any sample is not sufficient to allow use of the recommended 1000 ng of DNA, use the full remaining volume of DNA sample (approximately 10 to 12 µL, containing at least 500 ng) for the hybridization step.

The automation protocol **06a Aliquot_Libraries** is used to prepare a new sample plate containing the appropriate quantity of each DNA sample for hybridization. Before running the automation protocol, you must create a table containing instructions for the NGS Bravo indicating the volume of each sample to aliquot, as described in the steps below.

Prepare .csv file for sample normalization and aliquoting

2 Create a .csv (comma separated value) file with the headers shown in **Figure 10**. 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.

3 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 (see **page 80** for guidelines). 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	5.35
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 10 Sample spreadsheet for 1-column run

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option A\XT_HS2_ILM_v.Ax.x.x\Aliquot Input File Templates\Aliquot_Libraries_Template.csv** (where x.x.x is the version number).

The Aliquot_Libraries_template.csv file may be copied and used as a template for creating the .csv files 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.

4 Load the .csv file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option A\XT_HS2_ILM_v.Ax.x.x\Aliquot Input File Templates**.

Prepare the NGS Bravo and reagents for protocol 06a Aliquot_Libraries

5 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.

6 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.

7 Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water. If available, use the same Agilent shallow well reservoir that was used in the Cleanup_Pre-CapPCR protocol (04a or 04b).

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, you may retain the Agilent shallow well reservoir for use in the **09 SSELWash_XT_HS2_ILM** protocol (see **"Step 4. Wash the captured DNA"** on page 107) if you are running that protocol today.

Load the NGS Bravo

8 Load the Bravo deck according to **Table 47**.

Table 47 Initial Bravo deck configuration for 06a Aliquot_Libraries protocol

Location	Content
2	Nuclease-free water reservoir from **step 7**
5	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
6	Empty tip box
8	New, unused tip box*
9	Prepped library DNA in destination plate (Eppendorf twin.tec or Armadillo plate)

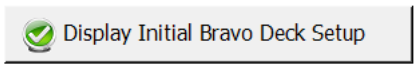
* The **06a Aliquot_Libraries** protocol does not use the Current Tip State indicator. The tip box at position 8 must be new and full of unused tips.

Run VWorks protocol 06a Aliquot_Libraries

9 On the SureSelect setup form, under **Select protocol to execute**, select the **06a Aliquot_Libraries** protocol.

10 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

11 Click **Display Initial Bravo Deck Setup**.



12 Verify that the Bravo deck has been set up as displayed on the right side of the form.

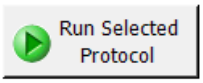
13 Upload the .csv file created in **step 2** through **step 4**.

a Click the "..." button below **Select Aliquot Input File** 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 below **Select Aliquot Input File**.

14 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.

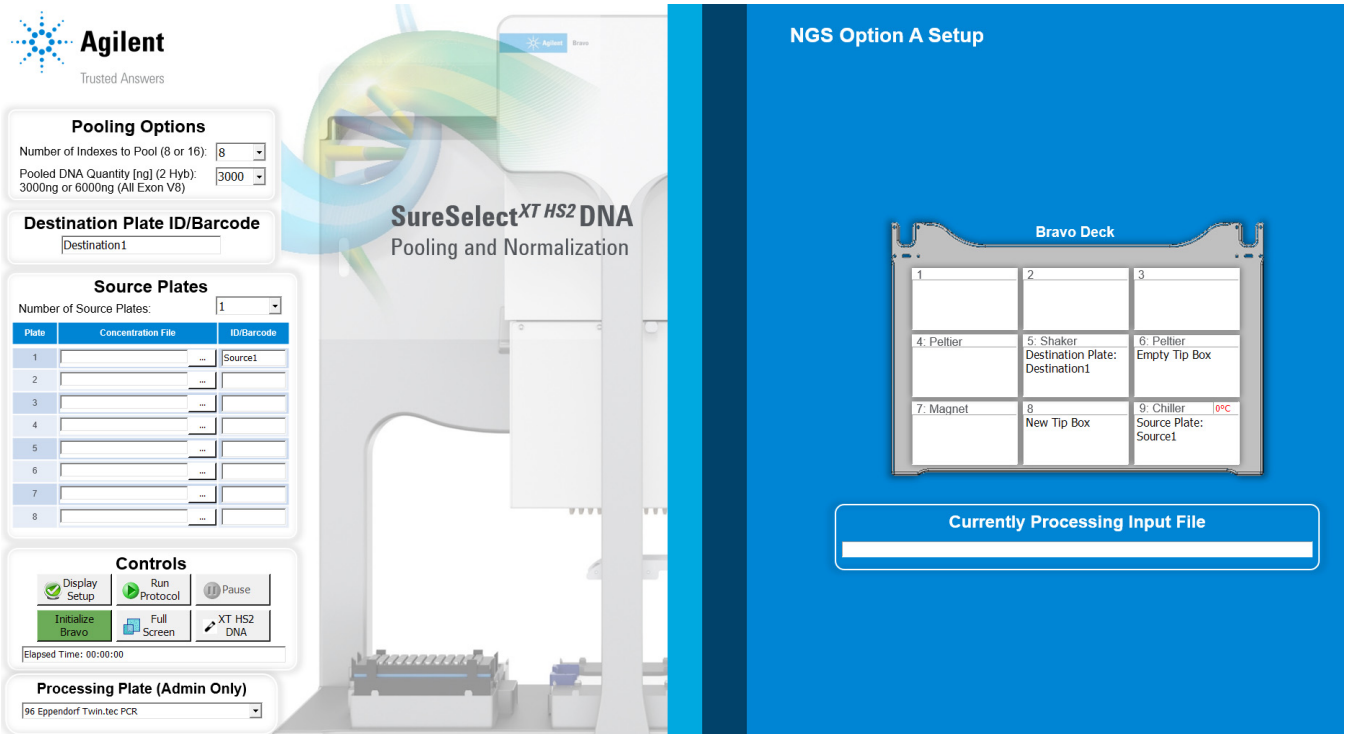
15 Remove the sample plate from the Bravo deck. Proceed directly to **"Step 2. Hybridize the gDNA library or library pool and probe"** on page 93.

Option 2: Multi-plex hybridization

This step uses automation protocols **PreCapture_Pooling** (accessed from the XT_HS2_Pooling VWorks form), **06b Aliquot_Water**, and **06c PoolingConcentration_XT_HS2_ILM**.

- The **PreCapture_Pooling** protocol pools the prepped indexed gDNA samples using automation protocol **PreCapture_Pooling**, which is set up using the XT_HS2_Pooling VWorks form, shown below. This form is accessible from within the XT_HS2_ILM form.
- The **06b Aliquot_Water** protocol adds enough water to each DNA library pool to bring the volume to 100 µL.
- Then, **06c PoolingConcentration_XT_HS2_ILM** protocol uses AMPure XP beads (prepared on **page 40**) to purify the DNA library pools, eluting the DNA in a volume of 24 µL.

Follow the steps in this section if you are using the pre-capture pooling workflow. If you are using the post-capture pooling workflow, see **"Option 1: Single-plex hybridization"** on page 80.



Plan pooling run parameters

The hybridization reaction requires 1500 ng indexed gDNA (or 3000 ng of indexed gDNA if using one of the SureSelect XT HS PreCap Human All Exon V8 Probes). The indexed gDNA is made up of a pool that contains equal amounts of 8 or 16 individual libraries. See **Table 48** for the recommended pool composition based on your SureSelect or ClearSeq Probe.

Table 48 Pre-capture pooling of indexed DNA libraries

Probe	Amount of total DNA per pool (Amount of DNA pool per hybridization reaction)*	Number of indexed gDNA libraries per pool	Amount of each indexed gDNA library in pool	Maximum DNA concentration for pool
SureSelect XT HS PreCap Human All Exon V8 Probe	6000 ng (3000 ng/hybridization)	8	750 ng	375 ng/µL
SureSelect XT HS PreCap Human All Exon V8+UTR Probe	6000 ng (3000 ng/hybridization)	8	750 ng	375 ng/µL
SureSelect XT HS PreCap Human All Exon V8+NCV Probe	6000 ng (3000 ng/hybridization)	8	750 ng	375 ng/µL
SureSelect Custom Probe	3000 ng (1500 ng/hybridization)	16	187.5 ng	93.75 ng/µL
ClearSeq Comprehensive Cancer	3000 ng (1500 ng/hybridization)	16	187.5 ng	93.75 ng/µL
SureSelect Human or Mouse All-Exon	3000 ng (1500 ng/hybridization)	8	375 ng	187.5 ng/µL
SureSelect Clinical Research Exome	3000 ng (1500 ng/hybridization)	8	375 ng	187.5 ng/µL
SureSelect Focused Exome	3000 ng (1500 ng/hybridization)	8	375 ng	187.5 ng/µL
ClearSeq Inherited Disease	3000 ng (1500 ng/hybridization)	8	375 ng	187.5 ng/µL

* Where possible, indexed DNA pools are prepared containing a total DNA amount that is enough for two hybridization reactions, i.e., 6000 ng for hybridizations with one of the SureSelect XT HS PreCap Human All Exon V8 Probes (V8, V8+UTR, or V8+NCV) and 3000 ng for all other probes. 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 48**, 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 48**, then prepare 3000 ng DNA pools (or a 6000 ng pool if using one of the SureSelect XT HS PreCap Human All Exon V8 Probes).
  - If at least one of the samples is above the maximum DNA concentration shown in **Table 48**, 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 200 ng/µL, then the final DNA pool will contain 400 ng of each indexed DNA. Next, determine the total amount of DNA per pool, based on the Probe size. Continuing with the same example, a Focused Exome capture pool would contain 8 × 400 ng, or 3200 ng DNA.

Plan destination indexed DNA pool sample plate configuration

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 **07 Hyb_XT_HS2_ILM** 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.

Prepare .csv files for pooling and normalization

Before starting the sample 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 Bravo calculates the volume of each sample required to prepare each concentration-normalized pool for the hybridization step.

See **Figure 11** for required .csv file content. Pooling and normalization .csv file templates are provided in the following directory: **C:\VWorks Workspace\NGS Option A\XT_HS2_ILM_v.Ax.x.x\Pooling and Normalization Templates**.

1. 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.
2. 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 11 Sample pooling and normalization .csv file content

3 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 72** 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 84** for hybridization sample pool placement considerations.

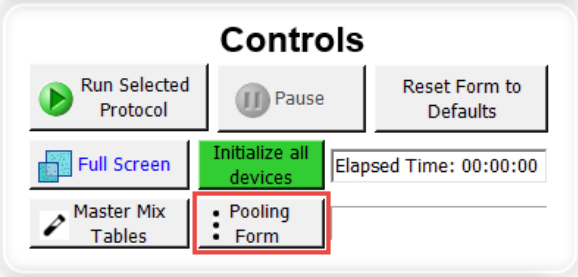
Set up and run the PreCapture_Pooling automation protocol

1 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.

2 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.

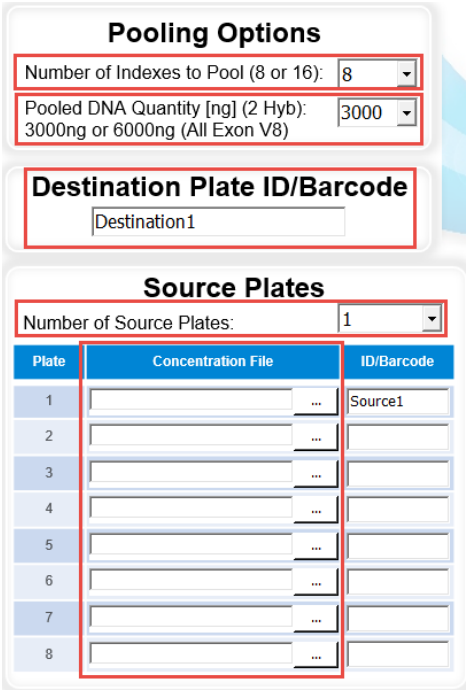
3 To set up the **PreCapture_Pooling** automation protocol, open the VWorks Form XT2_HS2_Pooling using one of the methods below.

- Double-click the shortcut on your desktop for the XT2_HS2_Pooling VWorks Form.
- In the directory **C:\VWorks Workspace\NGS Option A\XT_HS2_ILM_v.Ax.x.x\Forms** (where x.x.x is the version number) open the file **XT_HS2_Pooling_v.Ax.x.x.VWForm**.
- From the XT_HS2_ILM VWorks Form, under **Controls**, click **Pooling Form.**



4 In the XT2_HS2_Pooling Form, enter the run information highlighted below:

- Under **Pooling Options**, from **Number of Indexes to Pool** menu, select 8 or 16 (see **Table 48** on page 84 for guidelines).
- Under **Pooling Options**, from **Pooled DNA Quantity** menu, enter the required total amount of DNA in the pool. For the SureSelect XT HS Human All Exon V8 Probes, the required amount is 6000 ng. For all other Probes, the typical amount is 3000 ng. These amounts are sufficient for two hybridization reactions. See **page 83** for guidelines.
- Under **Destination Plate ID/Barcode**, enter the name or barcode of the destination plate into the field provided.
- Under **Source Plates**, from **Number of Source Plates** menu, 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 and normalization protocol in sets of 8 source plates.
- In the table under **Source Plates**, in **Concentration File** field, use the browse button to specify the location of each .csv file that provides sample position and concentration data for each plate.

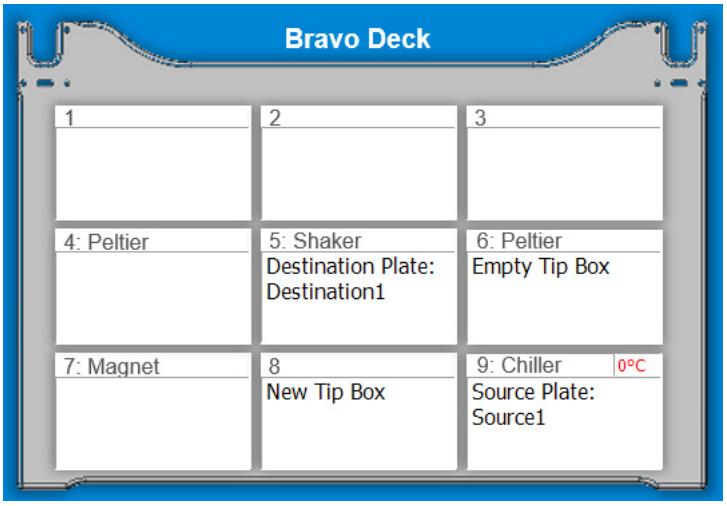


5 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

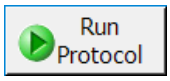
6 When finished entering run parameters in the Form, click **Display Setup**.

7 Load sample plates and labware as displayed on the right side of the form:

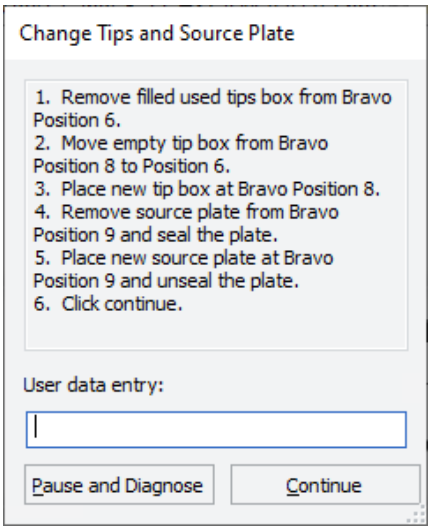
- Load the first indexed DNA source plate on Bravo deck position 9. (If you are processing more than one source plate, you are prompted to load the subsequent source plates during the **PreCapture_Pooling** protocol.)
- Load an empty destination plate (Eppendorf twin.tec or Armadillo plate, as indicated in the Processing Plate setting on the form) on Bravo deck position 5.
- Load an empty tip box on Bravo deck position 6.
- Load a new tip box on Bravo deck position 8.



8 When verification is complete, click **Run Protocol**.



CAUTION When more than one indexed DNA source plate is used in the run, an operator must be present during the run to remove and replace tip boxes and source plates during the run, in response to NGS Bravo prompts like the one shown below.

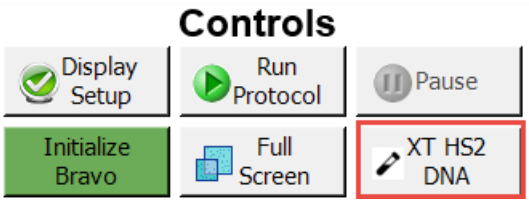


Running the **PreCapture_Pooling** protocol takes approximately one hour per indexed DNA source plate. Once complete, the destination sample plate, containing indexed DNA pools, is located at position 5 of the Bravo deck.

9 Remove the destination plate from Bravo deck position 5.

10 Seal the destination 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.

11 In the XT_HS2_Pooling VWorks form, under **Controls**, click **XT HS2 DNA** to return to the XT_HS2_ILM VWorks form. The remaining automation protocols are executed from the XT_HS2_ILM VWorks form.



Prepare .csv file for normalizing pooled DNA sample volumes to 100 µL

12 Create a .csv (comma separated value) file with the headers shown in **Figure 12**. 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.

13 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 12 Sample Aliquot_Water .csv file content

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option A\XT_HS2_ILM_v.Ax.x.x\Aliquot Input File Templates** (where x.x.x is the version number).

The Aliquot_Water_Template.csv file may be copied and used as a template for creating the .csv file for each **06b 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.

14 Load the .csv file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\XT_HS2_ILM_v.Bx.x.x\Pooling and Normalization Templates**.

Prepare the NGS Bravo and reagents for protocol 06b Aliquot_Water

15 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.

16 Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water. If available, use the same Agilent shallow well reservoir that was used in the Cleanup_Pre-CapPCR protocol (04a or 04b).

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, you may retain the Agilent shallow well reservoir for use in the **06c PoolingConcentration_XT_HS2_ILM** protocol if you are running that protocol today.

Load the NGS Bravo

17 Load the Bravo deck according to **Table 49**.

Table 49 Initial Bravo deck configuration for 06b Aliquot_Water protocol

Location	Content
5	Pooled library DNA in processing plate (Eppendorf twin.tec or Armadillo plate)
6	Empty tip box
8	New, unused tip box*
9	Nuclease-free water reservoir from **step 16**

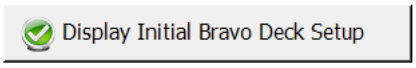
* The **06b Aliquot_Water** protocol does not use the Current Tip State indicator. The tip box at position 8 must be new and full of unused tips.

Run VWorks protocol 06b Aliquot_Water

18 On the SureSelect setup form, under **Select protocol to execute**, select the **06b Aliquot_Water** protocol.

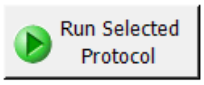
19 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

20 Click **Display Initial Bravo Deck Setup**.



21 Verify that the Bravo deck has been set up as displayed on the right side of the form.

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



23 When prompted, browse to the .csv file created for the source plate of the current run in **step 4**, and then click **OK** to start the run.

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

24 Remove the sample plate from the Bravo deck.

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

Prepare the NGS Bravo and reagents for protocol 06c PoolingConcentration_XT_HS2_ILM

26 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.

27 Pre-set the temperature of Bravo deck position 4 to 45°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.

28 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.

29 Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water. If available, use the same Agilent shallow well reservoir that was used in the **06b Aliquot_Water** protocol.

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

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

Load the NGS Bravo

31 Load the Bravo deck according to **Table 50**.

Table 50 Initial Bravo deck configuration for 06c PoolingConcentration_XT_HS2_ILM protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
3	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
5	Aliquotted AMPure XP beads in Agilent deep well plate from **page 40** (180 µL of beads/well)
6	Eppendorf twin.tec or Armadillo plate containing DNA library pools from the **06b Aliquot_Water** protocol
8	Empty tip box
9	70% ethanol reservoir from **step 30**

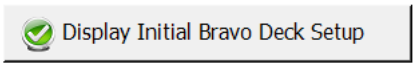
Run VWorks protocol 06c PoolingConcentration_XT_HS2_ILM

32 On the SureSelect setup form, under **Select protocol to execute**, select the **06c PoolingConcentration_XT_HS2_ILM** protocol.

33 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

34 Select the number of columns of samples to be processed.

35 Click **Display Initial Bravo Deck Setup**.



36 Verify that the Bravo deck has been set up as displayed on the right side of the form.

37 Upload the .csv file created in **step 12** through **step 14**.

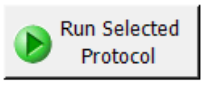
a Click the "..." button below **Select Aliquot Input File** to open a directory browser window.

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

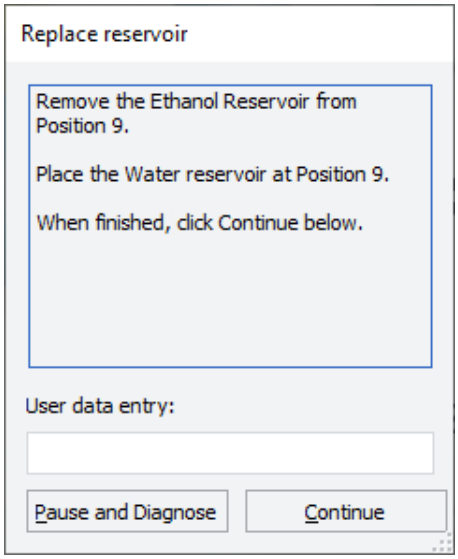
The directory browser window closes, returning you to the SureSelect setup form. The selected file location is listed in the field below **Select Aliquot Input File**.

38 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



The **06c PoolingConcentration_XT_HS2_ILM** protocol takes approximately 40 minutes. An operator must be present during the run to transfer the water reservoir to the Bravo deck, when directed by the VWorks prompt shown below.



When complete, the purified, pooled DNA samples are in the Eppendorf twin.tec or Armadillo plate located on Bravo deck position 3.

Step 2. Hybridize the gDNA library or library pool and probe

This step uses automation protocol **07 Hyb_XT_HS2_ILM**.

In this step, the NGS Bravo completes the liquid handling steps to set up the hybridization reactions. Afterward, you transfer the sample plate to a thermal cycler, held at 65°C, to allow hybridization of the DNA samples to the probe.

This step uses the components listed in **Table 51**. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 51 Reagents for Hybridization

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect XT HS2 Blocker Mix (blue cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	**page 95**
SureSelect RNase Block (purple cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	**page 96**
SureSelect Fast Hybridization Buffer (bottle)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw and keep at Room Temperature	**page 96**
Probe	–80°C	Thaw on ice	**page 96**

Pre-program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with the appropriate program for the Probe.
   - For the SureSelect XT HS Human All Exon V8 Probes and SureSelect XT HS PreCap Human All Exon V8 Probes – including V8, V8+UTR, and V8+NCV – use the program in **Table 52**.
   - For all other probes, use the program in **Table 53**.

2 Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

   It is critical to pre-program the thermal cycler before starting the automation protocol for hybridization, in order to maintain the required sample and reagent temperatures during the workflow.

Table 52 Hybridization program for *SureSelect XT HS and PreCap XT HS Human All Exon V8 Probes**

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 Bravo steps†	1	65°C	Hold
4	Hybridization	60	65°C	1 minute
			37°C	3 seconds
5	Hybridization	1	65°C	60 minutes
6	Hold until start of Capture‡	1	65°C†	Hold

* When setting up the thermal cycler program, use a reaction volume setting of 35 µL (final volume of hybridization reactions during cycling in Segment 4).

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

‡ Samples are held at 65°C until they are processed in the Capture & Wash automation protocol that begins on **page 103**.

Table 53 Hybridization program for *all other probes**

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 Bravo steps†	1	65°C	Hold
4	Hybridization	60	65°C‡	1 minute
			37°C	3 seconds
5	Hold until start of Capture**	1	65°C†	Hold

* When setting up the thermal cycler program, use a reaction volume setting of 35 µL (final volume of hybridization reactions during cycling in Segment 4).

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

‡ Hybridization at 65°C is optimal for probes designed for the SureSelect XT HS2/XT HS/XT Low Input platforms. Reducing the hybridization temperature (Segments 4 and 5) may improve performance for probes designed for the SureSelect XT platform, including SureSelect XT Human All Exon V6 (62.5°C), SureSelect XT Clinical Research Exome V2 (62.5°C) and custom probes originally designed for use with SureSelect XT system (60°C–65°C).

** Samples are held at 65°C until they are processed in the Capture & Wash automation protocol that begins on **page 103**.

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

NOTE

The Hybridization reaction may be run overnight with the following protocol modification.

- In the final segment of the thermal cycler program (**Table 52** or **Table 53**), replace the 65°C Hold step with a 21°C Hold step.

The hybridized samples may be held at 21°C for up to 16 hours.

Prepare the NGS Bravo for protocol 07 Hyb_XT_HS2_ILM

3 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.

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 Pre-set the temperature of Bravo deck positions 4 and 6 to 23°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. 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 4.

Prepare the Block master mix

7 Prepare the appropriate volume of Block master mix, on ice, as indicated in **Table 54**. Mix by vortexing at medium speed for 15–20 seconds. Keep on ice.

Table 54 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 54 Preparation of Block master mix

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	2.5 µL	31.9	53.1	74.4	95.6	116.9	138.1	161.1	184.2	207.2	230.2	253.2	276.3
SureSelect XT HS2 Blocker Mix (blue cap)	5.0 µL	63.8	106.3	148.8	191.3	233.8	276.3	322.3	368.3	414.4	460.4	506.5	552.5
Total Volume	**7.5 µL**	**95.7**	**159.4**	**223.2**	**286.9**	**350.7**	**414.4**	**483.4**	**552.5**	**621.6**	**690.6**	**759.7**	**828.8**

Prepare one or more Probe Hybridization master mixes

8 Prepare the appropriate volume of Probe Hybridization master mix for each of the Probes that will be used for hybridization as indicated in **Table 55** to **Table 58**. Mix by vortexing at medium speed for 15–20 seconds, then spin down briefly. Keep the Probe 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 55** or **Table 56**) on **page 96**.

For runs that use different Probes for individual rows, prepare each master mix as described in Step b (**Table 57** or **Table 58**) on **page 97**.

a For runs that use a single Probe for all rows, prepare a master mix as described in **Table 55** or **Table 56**, according to the probe design size.

Table 55 Preparation of Probe Hybridization master mix for Probes <3 Mb, 8 rows of wells

Reagent	Volume for 1 Library	Target size <3.0 Mb Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	7.0 µL	89.3	148.8	208.3	267.8	327.3	401.6	471.0	540.5	609.9	679.3	748.7	818.1
RNase Block (purple cap)	0.5 µL	6.4	10.6	14.9	19.1	23.4	28.7	33.6	38.6	43.6	48.5	53.5	58.4
SureSelect Fast Hybridization Buffer	6.0 µL	76.5	127.5	178.5	229.5	280.5	344.3	403.8	463.3	522.8	582.3	641.8	701.3
Probe (with design <3.0 Mb)	2.0 µL	25.5	42.5	59.5	76.5	93.5	114.8	134.6	154.4	174.3	194.1	213.9	233.8
Total Volume	**15.5 µL**	**197.7**	**329.4**	**461.2**	**592.9**	**724.7**	**889.4**	**1043.0**	**1196.8**	**1350.6**	**1504.2**	**1657.9**	**1811.6**

Table 56 Preparation of Probe Hybridization master mix for Probes ≥3 Mb, 8 rows of wells

Reagent	Volume for 1 Library	Target size ≥3.0 Mb Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	4.0 µL	51.0	85.0	119.0	153.0	187.0	229.5	269.2	308.8	348.5	388.2	427.8	467.5
RNase Block (purple cap)	0.5 µL	6.4	10.6	14.9	19.1	23.4	28.7	33.6	38.6	43.6	48.5	53.5	58.4
SureSelect Fast Hybridization Buffer	6 µL	76.5	127.5	178.5	229.5	280.5	344.3	403.8	463.3	522.8	582.3	641.8	701.3
Probe (with design ≥3.0 Mb)	5.0 µL	63.8	106.3	148.8	191.3	233.8	286.9	336.5	386.0	435.6	485.2	534.8	584.4
Total Volume	**15.5 µL**	**197.7**	**329.4**	**461.2**	**592.9**	**724.7**	**889.4**	**1043.1**	**1196.7**	**1350.5**	**1504.2**	**1657.9**	**1811.6**

b For runs that use different Probes in individual rows, prepare a master mix for each Probe as listed in **Table 57** or **Table 58**, according to the probe design size. The volumes listed in **Table 57** and **Table 58** 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 57 Preparation of Probe Hybridization master mix for Probes <3 Mb, single row of wells

Reagent	Volume for 1 Library	Target size <3.0 Mb Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	7	10.5	17.5	24.5	31.5	38.5	49.0	57.2	65.3	73.5	81.7	89.8	98.0
RNase Block (purple cap)	0.5	0.8	1.3	1.8	2.3	2.8	3.5	4.1	4.7	5.3	5.8	6.4	7.0
SureSelect Fast Hybridization Buffer	6.0	9.0	15.0	21.0	27.0	33.0	42.0	49.0	56.0	63.0	70.0	77.0	84.0
Probe (with design <3 Mb)	2.0	3.0	5.0	7.0	9.0	11.0	14.0	16.3	18.7	21.0	23.3	25.7	28.0
Total Volume	**15.5**	**23.3**	**38.8**	**54.3**	**69.8**	**85.3**	**108.5**	**126.6**	**144.7**	**162.8**	**180.8**	**198.9**	**217.0**

Table 58 Preparation of Probe Hybridization master mix for Probes ≥3 Mb, single row of wells

Reagent	Volume for 1 Library	Target size ≥3.0 Mb Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	4.0	6.0	10.0	14.0	18.0	22.0	28.0	32.7	37.3	42.0	46.7	51.3	56.0
RNase Block (purple cap)	0.5	0.8	1.3	1.8	2.3	2.8	3.5	4.1	4.7	5.3	5.8	6.4	7.0
SureSelect Fast Hybridization Buffer	6.0	9.0	15.0	21.0	27.0	33.0	42.0	49.9	56.0	63.0	70.0	77.0	84.0
Probe (with design ≥3 Mb)	5.0	7.5	12.5	17.5	22.5	27.5	35.0	40.8	46.7	52.5	58.3	64.2	70.0
Total Volume	**15.5**	**23.3**	**38.8**	**54.3**	**69.8**	**85.3**	**108.5**	**126.6**	**144.7**	**162.8**	**180.8**	**198.9**	**217.0**

Prepare the master mix source plate

9 Prepare the hybridization master mix source plate at room temperature, containing the master mixes prepared in **step 7** and **step 8**. Add the volumes indicated in **Table 59** 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 processing plate. The final configuration of the master mix source plate is shown in **Figure 13**.

Use either an **Eppendorf twin.tec** plate or an **Armadillo** plate as the master mix source plate, as indicated in the Processing Plate setting on the form.

Table 59 Preparation of the master mix source plate for 07 Hyb_XT_HS2_ILM protocol

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Block master mix	Column 1 (A1-H1)	11.0	19.0	27.0	34.9	42.9	50.9	59.5	68.1	76.8	85.4	94.0	102.7
Probe Hybridization master mix	Column 2 (A2-H2)	23.3	38.8	54.3	69.8	85.3	108.5	126.6	144.7	162.8	180.8	198.9	217.0*

* Wells containing 217.0 µL are nearly full. Pipette carefully to avoid introducing bubbles to the bottom of the wells. Handle the plate with care to avoid spillage. Do not centrifuge.

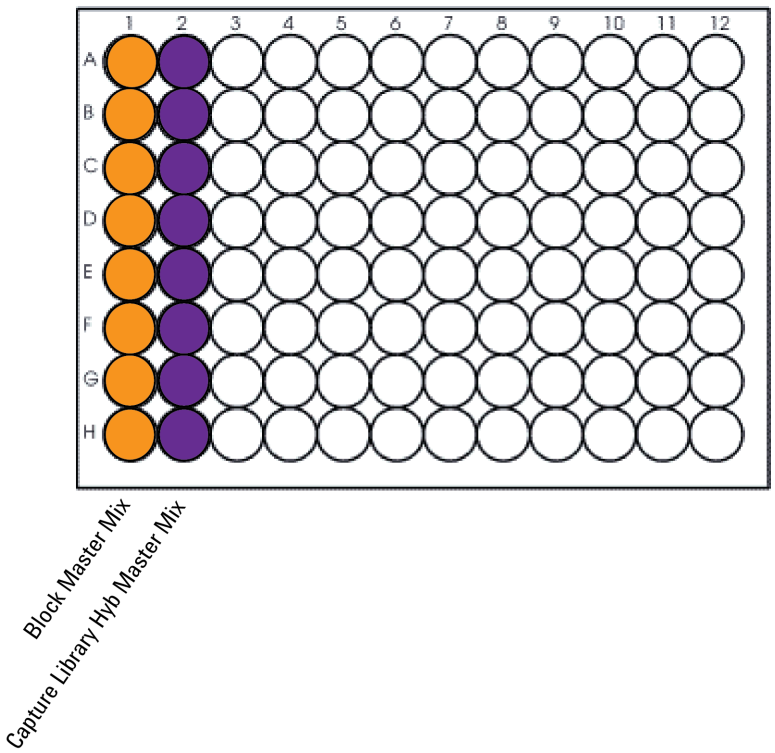


Figure 13 Configuration of the master mix source plate for protocol **07 Hyb_XT_HS2_ILM**. Column 2 can contain different Probe Hybridization master mixes in each row.

10 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

11 Load the Bravo deck according to **Table 60**.

Table 60 Initial Bravo deck configuration for Hyb_XT_HS2_ILM protocol

Location	Content
2	New tip box
4	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
5	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
6	Master Mix source plate (unsealed)
8	Empty tip box
9	Prepared library aliquots or library pools in Eppendorf twin.tec or Armadillo plate (unsealed)

Run VWorks protocol Hyb_XT_HS2_ILM

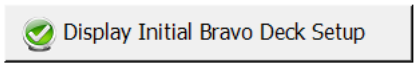
12 On the SureSelect setup form, under **Select protocol to execute**, select the **07 Hyb_XT_HS2_ILM** protocol.

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

14 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

15 Select the number of columns of samples to be processed.

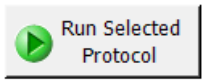
16 Click **Display Initial Bravo Deck Setup**.



17 Verify that the Bravo deck has been set up as displayed on the right side of the form.

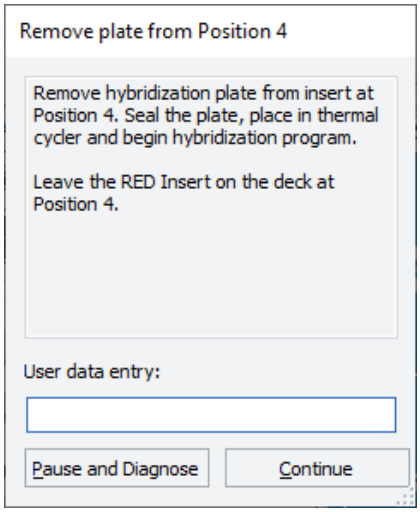
18 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



The NGS Bravo combines the prepped gDNA in the wells of the sample plate with the aliquotted SureSelect Block master 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.

20 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**.



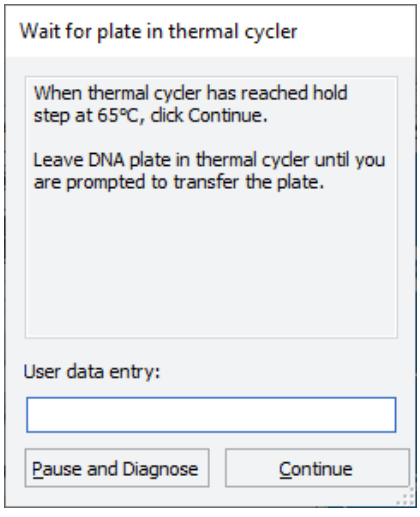
21 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

22 Transfer the sealed plate to a thermal cycler. Initiate the pre-programmed thermal cycler program (**Table 52** on page 94 or **Table 53** on page 94).

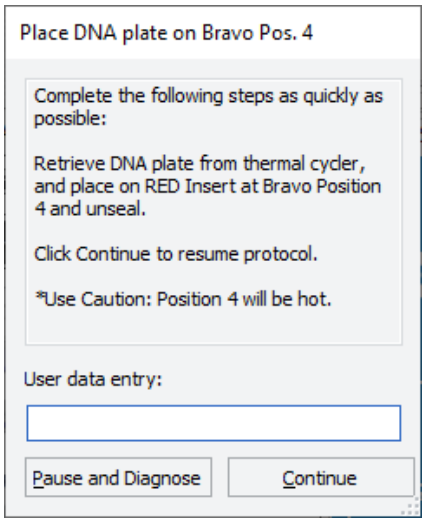
While the sample plate incubates on the thermal cycler, the NGS Bravo aliquots the Probe Hybridization master mix to the Eppendorf twin.tec or Armadillo plate.

CAUTION You must complete **step 23** to **step 27** 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 Bravo and thermal cycler.

23 When the NGS Bravo has finished aliquoting the Probe 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.



24 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**.

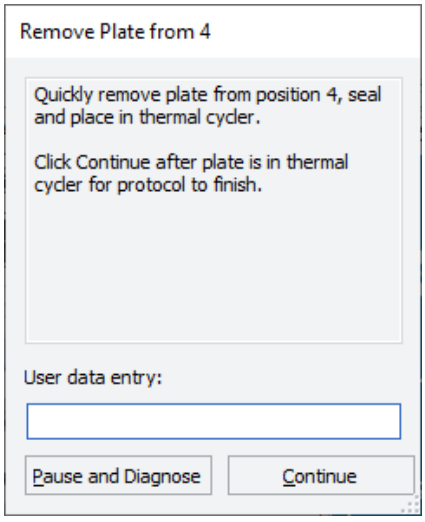


WARNING **Bravo deck position 4 will be hot.**

Use caution when handling components that contact heated deck positions.

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

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



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

27 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 52** on page 94 or **Table 53** on page 94). During this step, the prepared DNA samples or DNA sample pools are hybridized to the Probe.

CAUTION 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.

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

29 If you are using the pre-capture pooling workflow, when the Hybridization protocol is complete, remove the Eppendorf twin.tec or Armadillo 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.

Retain the Eppendorf twin.tec or Armadillo source plate containing the Block master mix and Probe Hybridization master mix located at position 6 of the Bravo deck for later use in the **10 Post-CapPCR_XT_HS2_ILM** protocol.

Step 3. Capture the hybridized DNA

This step uses automation protocol **08 SSELCapture_XT_HS2_ILM**.

In this step, the NGS Bravo automates capture of the gDNA-probe hybrids using streptavidin-coated magnetic beads.

If performing same-day hybridization and capture, setup tasks for the Capture protocol (**step 1**, below, through **step 18** on **page 105**) should be completed during the thermal cycler incubation for hybridization (approximately 1.5 to 2.5-hour duration) started on **page 102**. If performing next-day capture after an overnight hold at 21°C, begin these tasks on day 2.

This step uses the components listed in **Table 61**.

Table 61 Reagents for Capture

Kit Component	Storage Location	Where Used
SureSelect Binding Buffer	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	**page 104**
SureSelect Wash Buffer 1	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	**page 104**
SureSelect Wash Buffer 2	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	**page 104**
Dynabeads MyOne Streptavidin T1	Follow storage recommendations provided by supplier (see **Table 1** on page 11)	**page 104**

Prepare the NGS Bravo for protocol 08 SSELCapture_XT_HS2_ILM

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Place a red PCR plate insert at Bravo deck position 4.
3. Place the silver Deep Well plate insert on position 6 of the Bravo deck. This insert is required to facilitate heat transfer to the Deep Well source plate wells. When loading a source plate on the silver insert, make sure the plate is seated properly to ensure proper heat transfer.

Prepare the Dynabeads streptavidin beads source plate

4 Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. The beads settle during storage.

5 Wash the magnetic beads.

a In a conical vial, combine the components listed in **Table 62**. The volumes below include the required overage.

Table 62 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 62 Magnetic bead washing mixture

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Dynabeads MyOne Streptavidin T1 bead suspension	50 µL	425	825	1225	1650	2050	2500	2900	3350	3750	4200	4600	5000
SureSelect Binding Buffer	200 µL	1700	3300	4900	6600	8200	10000	11600	13400	15000	16800	18400	20000
Total Volume	**250 µL**	**2125**	**4125**	**6125**	**8250**	**10260**	**12500**	**14500**	**16750**	**18750**	**21000**	**23000**	**25000**

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

c Put the vial 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.)

6 Resuspend the beads in SureSelect Binding buffer, according to **Table 63** below.

Table 63 Resuspension volumes for washed bead suspension

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
SureSelect Binding Buffer	200 µL	1700	3300	4900	6600	8200	10000	11600	13400	15000	16800	18400	20000

7 Prepare an Agilent Deep Well source plate for the washed streptavidin bead suspension. For each well to be processed, add 200 µL of the homogeneous bead suspension to the Agilent Deep Well plate.

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

Prepare wash buffer source plates

9 Prepare a processing source plate (Eppendorf twin.tec or Armadillo plate) labeled *Wash #1*. For each well to be processed, add 150 µL of SureSelect Wash Buffer 1.

10 Prepare an Agilent Deep Well source plate labeled *Wash #2*. For each well to be processed, add 1150 µL of SureSelect Wash Buffer 2.

Load the NGS Bravo

11 Load the Bravo deck according to **Table 64** (position 5 should already be loaded).

Table 64 Initial Bravo deck configuration for 08 SSELCapture_XT_HS2_ILM protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
3	*Wash #1* source plate (Eppendorf twin.tec or Armadillo plate) from **step 9**
4	Empty red insert
5	Dynabeads streptavidin bead Deep Well source plate
6	*Wash #2* source plate (Agilent Deep Well plate) from **step 10** seated on silver Deep Well insert
8	Empty tip box

Set up VWorks protocol 08 SSELCapture_XT_HS2_ILM

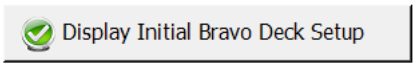
12 On the SureSelect setup form, under **Select protocol to execute**, select the **08 SSELCapture_XT_HS2_ILM** protocol.

13 Under **Select labware 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.

14 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

15 Select the number of columns of samples to be processed.

16 Click **Display Initial Bravo Deck Setup**.



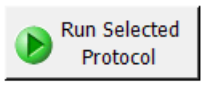
17 Verify that the Bravo deck has been set up as displayed on the right side of the form.

18 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

Run VWorks protocol 08 SSELCapture_XT_HS2_ILM

Start the **08 SSELCapture_XT_HS2_ILM** protocol upon completion of the hybridization incubation. The hybridization incubation is complete when the thermal cycler program reaches the 65°C Hold step. The 65°C Hold step is segment 6 if using **Table 52** on page 94, and it is segment 5 if using **Table 53** on page 94.

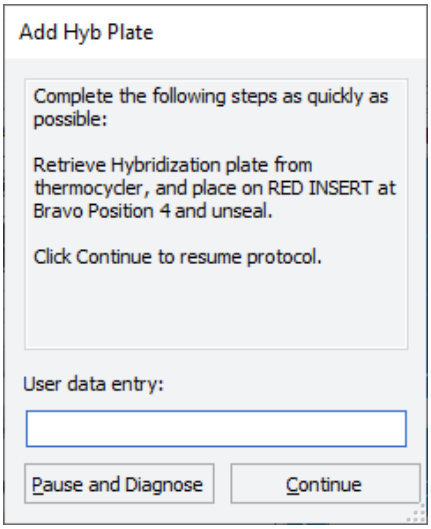
After verifying that the hybridization step is complete and that all NGS Bravo setup steps for capture are complete, click **Run Selected Protocol**. Leave the hybridization plate in the thermal cycler until you are prompted to transfer the plate to the NGS Bravo.



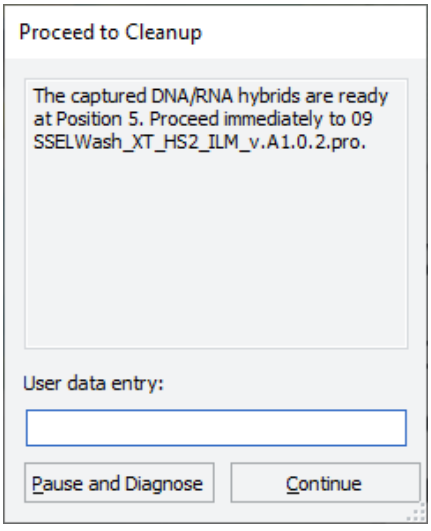
The total duration of the **08 SSELCapture_XT_HS2_ILM** protocol is approximately 35 minutes. An operator must be present to transfer the hybridization plate from the thermal cycler when prompted by VWorks as shown in **step 19** below (<5 minutes after starting the protocol).

CAUTION It is important to complete **step 19** quickly and carefully. Unseal the plate without tilting or jerking the plate to avoid sample splashing. Make sure that the NGS Bravo is completely prepared, with all components in place, before you transfer the sample plate to the Bravo deck.

19 When prompted by VWorks as shown below, quickly remove the PCR plate, containing the hybridization reactions held at 65°C, 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 protocol.



20 When the capture incubation period is complete you will be prompted by VWorks as shown below. Keep the hybrid-capture bead suspension plate at position 5 and proceed immediately to automation protocol **09 SSELWash_XT_HS2_ILM**.



Step 4. Wash the captured DNA

This step uses automation protocol **09 SSELWash_XT_HS2_ILM**.

In this step, the NGS Bravo automates washing of the captured DNA-RNA hybrids.

Prepare the NGS Bravo and reagents for protocol 09 SSELWash_XT_HS2_ILM

1 Keep the following contents from the previous automation protocol in their current positions on the Bravo deck.

- Position 3: Wash Buffer 1 source plate
- Position 4: Red PCR plate insert (empty)
- Position 5: Hybrid-capture bead suspension plate
- Position 6: Wash Buffer 2 source plate

Clear the remaining Bravo deck positions of all plates and tip boxes.

2 Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in **Table 65**. See **Setting the Temperature of Bravo Deck Heat Blocks** for more information on how to do this step.

Table 65 Bravo Deck Temperature Presets for protocol 09 SSELWash_XT_HS2_ILM

Bravo Deck Position	Temperature Preset	Preset Method
4	80°C	Inheco Multi TEC control touchscreen (CPAC 2-1)
6	85°C	Inheco Multi TEC control touchscreen (CPAC 2-2)

3 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, you may retain the Agilent shallow well reservoir for use in the **10 Post-CapPCR_XT_HS2_ILM** protocol if you are running that protocol today.

Load the NGS Bravo

4 Load the Bravo deck according to **Table 66** (positions 3, 4, 5, and 6 should already be loaded).

Table 66 Initial Bravo deck configuration for 09 SSELWash_XT_HS2_ILM

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
3	*Wash #1* source plate (Eppendorf twin.tec or Armadillo plate)
4	Empty red insert
5	DNA-RNA hybrids captured on streptavidin beads in Agilent Deep Well plate
6	*Wash #2* source plate (Agilent Deep Well plate) seated on silver Deep Well insert
8	Empty tip box
9	Nuclease-free water reservoir from **step 3**

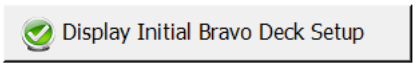
Run VWorks protocol 09 SSELWash_XT_HS2_ILM

5 On the SureSelect setup form, under **Select protocol to execute**, select the **09 SSELWash_XT_HS2_ILM** protocol.

6 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

7 Select the number of columns of samples to be processed.

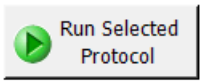
8 Click **Display Initial Bravo Deck Setup**.



9 Verify that the Bravo deck has been set up as displayed on the right side of the form.

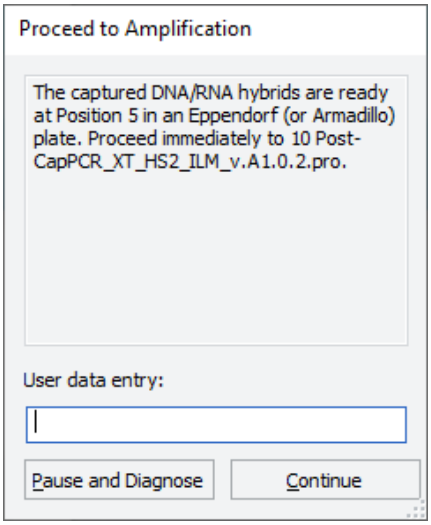
10 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

11 When setup and verification is complete, click **Run Selected Protocol**.



Running the **09 SSELWash_XT_HS2_ILM** protocol takes approximately 90 minutes. An operator must be present during the run to complete tip box replacement as directed by VWorks prompts. Once complete, you will be prompted as shown below.

12 When the wash protocol is complete, the captured, bead-bound DNA samples are located in the processing plate at position 5 of the Bravo deck, and you will be prompted by VWorks as shown below. Click **Continue** on the VWorks screen to finish the protocol.



Proceed immediately to the **10 Post-CapPCR_XT_HS2_ILM** protocol, starting on **page 112**.

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

6 Post-Capture Sample Processing for Multiplexed Sequencing

This chapter describes the steps to amplify, purify, and assess quality and quantity of the captured libraries. Sample pooling instructions for the post-capture pooling workflow are provided to prepare the indexed, molecular barcoded samples for multiplexed sequencing.

Step 1. Amplify the captured libraries

This step uses automation protocol **10 Post-CapPCR_XT_HS2_ILM**.

In this step, the NGS Bravo completes the liquid handling steps for PCR-amplification of the SureSelect-enriched DNA samples. After the PCR plate is prepared by the NGS Bravo, 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 69** on page 113 for cycle number recommendations.

This step uses the components listed in **Table 67**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 67 Reagents for post-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Pipette up and down 15–20 times	**page 113**
5× Herculase II Reaction Buffer with dNTPs (clear cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Vortexing	**page 113**
SureSelect Post-Capture Primer Mix (clear cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Vortexing	**page 113**

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 Bravo and reagents for protocol 10 Post-CapPCR_XT_HS2_ILM

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. 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.
3. 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 **09 SSELWash_XT_HS2** protocol.

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

Pre-program the thermal cycler

5 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 68**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

Table 68 Post-capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10 to 16 See **Table 69** for recommendations based on probe design size	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Table 69 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 Probes)	10–11 cycles

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

6 Prepare the appropriate volume of post-capture PCR Master Mix, according to **Table 70**. Mix well using a vortex mixer and keep on ice.

Table 70 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 70 Preparation of Post-Capture PCR master mix

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
5× Herculase II Reaction Buffer with dNTPs (clear cap)	10 µL	170	255	340	425	510	595	683.3	772.5	862.5	953.3	1045	1137.5
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	17.0	25.5	34.0	42.5	51.0	59.5	68.3	77.3	86.3	95.3	104.5	113.8
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17.0	25.5	34.0	42.5	51.0	59.5	68.3	77.3	86.3	95.3	104.5	113.8
Total Volume	**12.0 µL**	**204.0**	**306.0**	**408.0**	**510.0**	**612.0**	**714.0**	**819.9**	**927.1**	**1035.1**	**1143.9**	**1254.0**	**1365.1**

7 Using the same master mix source plate (Eppendorf twin.tec or Armadillo plate) that was used for the **07 Hyb_XT_HS2_ILM** protocol run, prepare the master mix source plate by adding the volume of PCR master mix indicated in **Table 71** to all wells of column 3 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 14**.

Table 71 Preparation of the master mix source plate for 10 Post-CapPCR_XT_HS2_ILM protocol

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Selected Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Post-Capture PCR Master Mix	Column 3 (A3-H3)	23.0	36.0	49.0	62.0	75.0	88.0	101.0	114.0	127.0	140.0	153.0	166.0

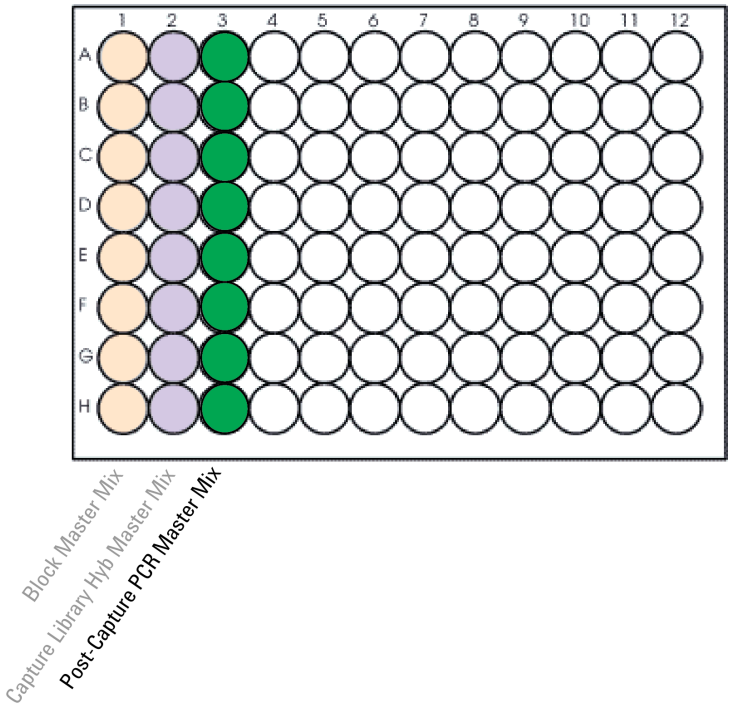


Figure 14 Configuration of the master mix source plate for protocol **10 Post-CapPCR_XT_HS2_ILM**. The master mixes dispensed during previous protocols are shown in light shading.

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

9 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 Bravo

10 Load the Bravo deck according to **Table 72**.

Table 72 Initial Bravo deck configuration for Post-CapPCR_XT_HS2_ILM protocol

Location	Content
1	Nuclease-free water reservoir from **step 3**
2	New tip box
5	Captured DNA bead suspensions in Eppendorf twin.tec or Armadillo plate (unsealed)
6	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
8	Empty tip box
9	Master mix source plate containing PCR Master Mix in Column 3 (unsealed)

Run VWorks protocol 10 Post-CapPCR_XT_HS2_ILM

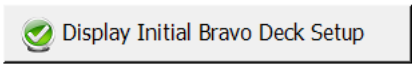
11 On the SureSelect setup form, under S**elect protocol to execute**, select the **10 Post-CapPCR_XT_HS2_ILM** protocol.

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

13 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

14 Select the number of columns of samples to be processed.

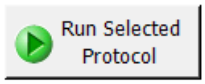
15 Click **Display Initial Bravo Deck Setup**.



16 Verify that the NGS Bravo has been set up as displayed on the right side of the form.

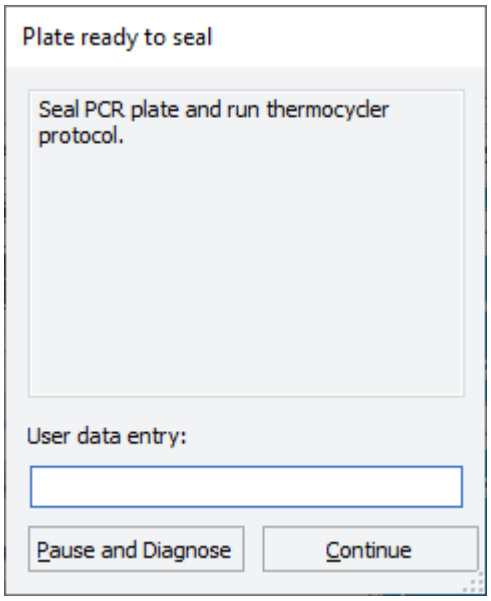
17 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **10 Post-CapPCR_XT_HS2_ILM** protocol takes approximately 10 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.

19 When you see the following prompt, remove the PCR plate from position 6 of the Bravo deck and seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 seconds.



20 Place the plate in the thermal cycler. Resume the thermal cycler program in **Table 68** on page 113.

21 When the PCR amplification program is complete, spin the plate briefly then keep on ice.

Retain the Eppendorf twin.tec or Armadillo source plate containing the Post-Capture PCR master mix located at position 9 of the Bravo deck for later use in the **12 TS_HighSensitivity_D1000** protocol (see **"Option 1: Analysis using the Agilent 4200 TapeStation and High Sensitivity D1000 ScreenTape"** on page 119).

Step 2. Purify the amplified indexed libraries using AMPure XP beads

This step uses automation protocol **11 Cleanup_Post-CapPCR_XT_HS2_ILM**.

In this step, the NGS Bravo transfers AMPure XP beads to the indexed DNA sample plate and then collects and washes the bead-bound DNA.

This step uses the aliquoted plate of AMPure XP beads that was prepared on **page 42**.

Prepare the NGS Bravo and reagents for protocol 11 Cleanup_Post-CapPCR_XT_HS2_ILM

1 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.

2 Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in **Table 73**. See **Setting the Temperature of Bravo Deck Heat Blocks** for more information on how to do this step.

Table 73 Deck Temperature Presets for protocol 11 Cleanup_Post-CapPCR_XT_HS2_ILM

Bravo Deck Position	Temperature Preset	Preset Method
4	45°C	Inheco Multi TEC control touchscreen (CPAC 2-1)
6	4°C	Inheco Multi TEC control touchscreen (CPAC 2-2)

3 Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water. If available, use the same Agilent shallow well reservoir that was used in the **10 Post-CapPCR_XT_HS2_ILM** protocol.

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

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

Load the NGS Bravo

5 Load the Bravo deck according to **Table 74**.

Table 74 Initial Bravo deck configuration for 11 Cleanup_Post-CapPCR_XT_HS2_ILM protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
3	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
5	Aliquotted AMPure XP beads in Agilent Deep Well plate from **page 42** (50 µL of beads/well)
6	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 2)

Table 74 Initial Bravo deck configuration for 11 Cleanup_Post-CapPCR_XT_HS2_ILM protocol

Location	Content
8	Empty tip box
9	70% ethanol reservoir from **step 4**

Run VWorks protocol 11 Cleanup_Post-CapPCR_XT_HS2_ILM

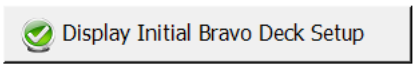
6 On the SureSelect setup form, under **Select protocol to execute**, select the **11 Cleanup_Post-CapPCR_XT_HS2_ILM** protocol.

7 Under **Select labware for thermal cycling**, select the specific type of PCR plate containing the amplified libraries at position 6.

8 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

9 Select the number of columns of samples to be processed.

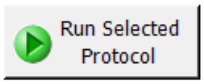
10 Click **Display Initial Bravo Deck Setup**.



11 Verify that the NGS Bravo has been set up as displayed on the right side of the form.

12 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



The purification protocol takes approximately 40 minutes. An operator must be present during the run to complete tip box replacement and to transfer the water reservoir to the Bravo deck, when directed by the VWorks prompt shown below.

When complete, the amplified DNA samples are in the processing plate located on Bravo deck position 3.

Step 3. 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 the Agilent 4200 TapeStation and High Sensitivity D1000 ScreenTape"** on page 119.
- Option 2: Prepare the analytical samples manually and perform analysis on Agilent 2100 Bioanalyzer, Agilent 4200 or 4150 TapeStation or Agilent 5200 Fragment Analyzer. See **"Option 2: Analysis using an equivalent platform (non-automated)"** on page 124.

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

This section describes use of automation protocol **12 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 either analysis system to equilibrate to room temperature for 30 minutes prior to use.

Prepare the NGS Bravo and Sample Buffer source plate for protocol 12 TS_HighSensitivity_D1000

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Turn off the ThermoCube device (see **page 22**) to restore position 9 of the Bravo deck to room temperature.
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. Using the same master mix source plate that was used for the **10 Post-CapPCR_XT_HS2_ILM** protocol run, prepare the Sample Buffer source plate at room temperature. Add the volume of High Sensitivity D1000 Sample Buffer indicated in **Table 75** to each well of column 4 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 15**.

Table 75 Preparation of the Sample Buffer Source Plate for 12 TS_HighSensitivity_D1000 protocol

Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Selected Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
High Sensitivity D1000 Sample Buffer	Column 4 (A4-H4)	8.0	11.0	14.0	17.0	20	23.0	26.5	30	33.5	37	40.5	44.0

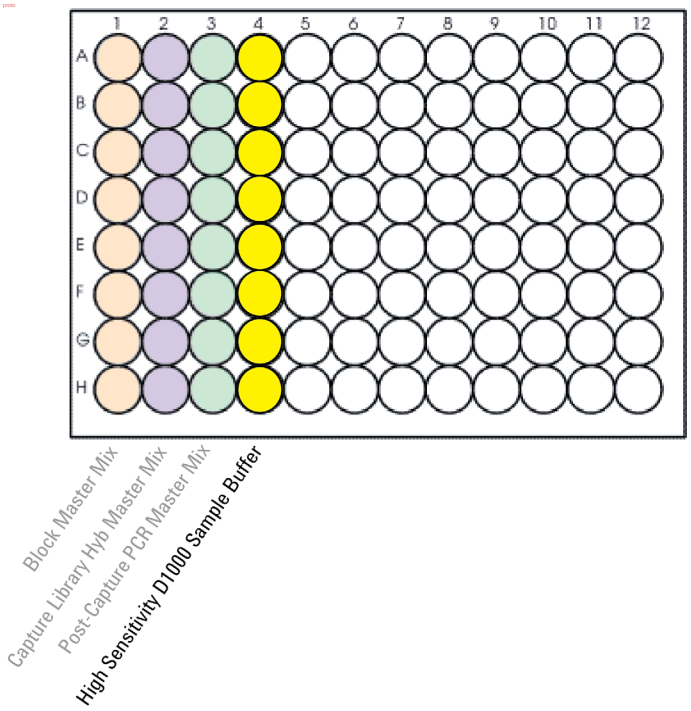


Figure 15 Configuration of the source plate for protocol **12 TS_HighSensitivity_D1000**. Columns 1–3 were used to dispense master mixes during previous protocols.

Load the NGS Bravo

5 Load the Bravo deck according to **Table 76**.

Table 76 Initial Bravo deck configuration for TS_HighSensitivity_D1000 protocol

Location	Content
2	New tip box
4	Amplified post-capture libraries or library pools in Eppendorf twin.tec or Armadillo plate (unsealed)
6	Empty TapeStation analysis plate (Agilent p/n 5042-8502)
8	Empty tip box
9	Master mix source plate (Eppendorf twin.tec or Armadillo plate) containing High Sensitivity D1000 Sample Buffer in Column 4

CAUTION To prevent damage to the Agilent 4200 TapeStation instrument and the NGS Bravo, use only the specified Agilent plates (Agilent p/n 5042-8502) for automated assay plate preparation.

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

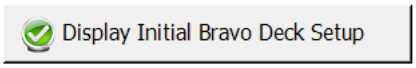
Run VWorks protocol 12 TS_HighSensitivity_D1000

6 On the SureSelect setup form, under **Select protocol to execute**, select the **12 TS_HighSensitivity_D1000** protocol.

7 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

8 Select the number of columns of samples to be processed.

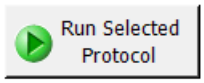
9 Click **Display Initial Bravo Deck Setup**.



10 Verify that the NGS Bravo has been set up as displayed on the right side of the form.

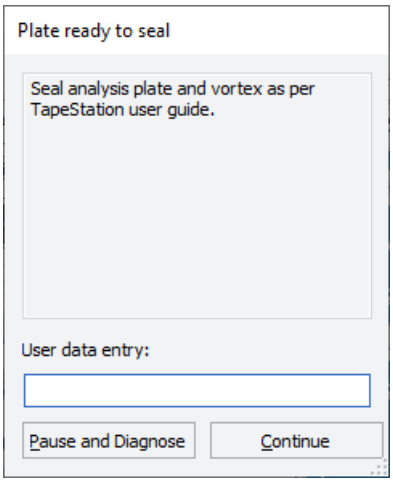
11 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **12 TS_HighSensitivity_D1000** protocol takes approximately 10 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 pooled for sequencing on **page 125**.

13 When prompted by VWorks as shown below, remove the TapeStation analysis 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

14 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.

15 Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 77** for guidelines). Sample electropherograms are shown in **Figure 16** (library prepared from high-quality DNA), **Figure 17** (library prepared from medium-quality FFPE DNA), and **Figure 18** (library prepared from low-quality FFPE DNA).

Table 77 Post-capture library qualification guidelines

NGS read length for fragmentation protocol selection	Input DNA type	Expected library DNA fragment size peak position
2 × 100 reads	Intact DNA	300 to 400 bp
	FFPE DNA	200 to 400 bp
2 × 150 reads	Intact DNA	330 to 450 bp
	FFPE DNA	200 to 450 bp

16 Determine the concentration of each library by integrating under the entire peak.

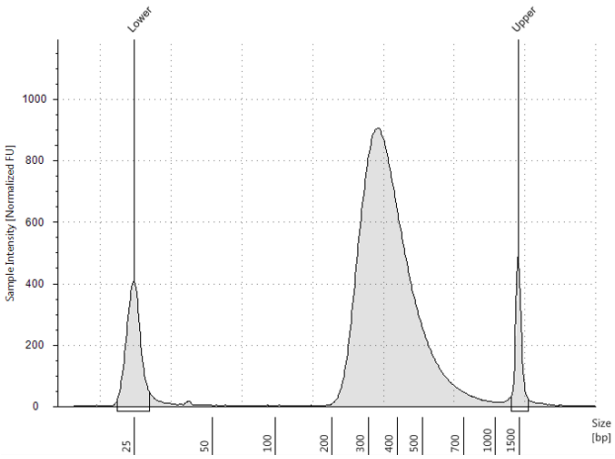


Figure 16 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

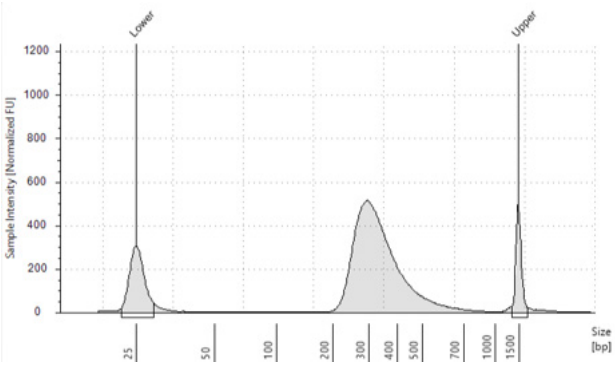


Figure 17 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

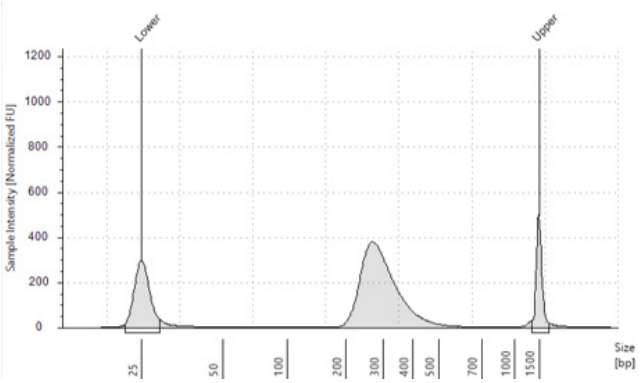


Figure 18 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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 Agilent 2100 BioAnalyzer, 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 16** through **Figure 18**). Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 77** for guidelines). **Table 78** includes links to assay instructions.

Table 78 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 2100 Bioanalyzer system	High Sensitivity DNA Kit	Agilent High Sensitivity DNA Kit Guide	1 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 4. Pool samples for multiplexed sequencing (optional)

NOTE

Pre-Capture Pooling Workflow If you are using the pre-capture pooling workflow, then your samples were already pooled prior to hybridization with the Probe. The final captured DNA samples contain pools of either 8 or 16 indexed libraries, based on the Probe used and resulting pre-capture pooling strategy. When appropriate for your sequencing platform, the 8-plex or 16-plex samples may be further multiplexed by post-capture pooling. Determine whether to do post-capture pooling by calculating the number of indexes that can be combined per lane, according to the output specifications of the platform used, together with the amount of sequencing data required for your research design. If doing post-capture pooling, use the guidelines provided below. If samples will not be further combined in post-capture pools, proceed to **"Step 5. Prepare sequencing samples"** on page 129.

Post-Capture Pooling Workflow For the post-capture pooling workflow, the number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample.

Combine the 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 **13 Aliquot_Captures** automation protocol to pool samples.

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) using Low TE. This dilution step is performed by manually pipetting the Low TE 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 **13 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 Low TE to each well. This volume adjustment is performed by manually pipetting the Low TE 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.

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

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 79 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 79 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 **12 Aliquot_Captures** automation protocol.

1 Create a .csv (comma separated value) file with the headers shown in **Figure 19**. 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 19** 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 19 Sample spreadsheets for method 1 and method 2

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option A\XT_HS2_ILM_v.Ax.x.x\Aliquot Input File Templates\Aliquot_Captures_Template.csv** (where x.x.x is the version number).

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 A\XT_HS2_ILM_v.Ax.x.x\Aliquot Input File Templates**.

4 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away 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.

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

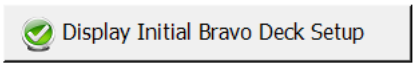
Table 80 Initial Bravo deck configuration for 13 Aliquot_Captures protocol

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 or Armadillo plate

7 On the SureSelect setup form, under **Select protocol to execute**, select the **13 Aliquot_Captures** protocol.

8 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

9 Click **Display Initial Bravo Deck Setup**.



10 Verify that the NGS Bravo has been set up as displayed on the right side of the form.

11 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

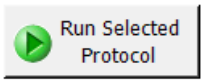
12 Upload the .csv file created in **step 1** through **step 3**.

a Click the "..." button below **Select Aliquot Input File** 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 below **Select Aliquot Input File**.

13 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.

14 Remove the Agilent Deep Well plate from position 5 of the Bravo deck.

15 Add the appropriate volume of Low TE to each well to bring the pool to the necessary DNA concentration for sequencing.

If you store the library before sequencing, add Tween 20 to 0.1% v/v and store at –20°C short term.

Step 5. Prepare sequencing samples

The final SureSelect XT HS2 library pool is ready for direct sequencing using standard Illumina paired-end primers and chemistry. Each fragment in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform, as shown in **Figure 20**.

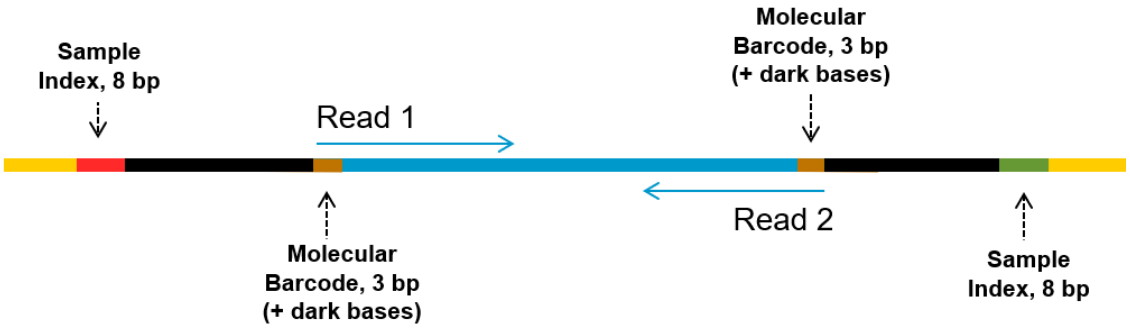


Figure 20 Content of SureSelect XT HS2 sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the dual sample indexes (red and green), molecular barcodes (brown) and the library PCR primers (yellow).

Libraries can be sequenced on the Illumina HiSeq, MiSeq, NextSeq or NovaSeq platform using the run type and chemistry combinations shown in **Table 81**.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See **Table 81** for kit configurations compatible with the recommended read length.

The optimal seeding concentration for SureSelect XT HS2 target-enriched libraries varies according to sequencing platform, run type, and Illumina kit version. See **Table 81** for guidelines. Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in **Table 81**.

Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Table 81 Illumina Kit Configuration Selection Guidelines

Platform	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
HiSeq 2500	Rapid Run	2 × 100 bp	200 Cycle Kit	v2	9–10 pM
HiSeq 2500	High Output	2 × 100 bp	250 Cycle Kit	v4	12–14 pM
MiSeq	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	9–10 pM
MiSeq	All Runs	2 × 75 bp	150 Cycle Kit	v3	12–16 pM
NextSeq 500/550	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2.5	1.2–1.5 pM
HiSeq 3000/4000	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1	300–400 pM
NovaSeq 6000	Standard Workflow Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1.0 or v1.5	300–600 pM
NovaSeq 6000	Xp Workflow Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1.0 or v1.5	200–400 pM

Step 6. Do the sequencing run and analyze the data

The guidelines below provide an overview of SureSelect XT HS2 library sequencing run setup and analysis considerations. Links are provided for additional details for various NGS platforms and analysis pipeline options.

- Each of the sample-level indexes requires an 8-bp index read. For complete index sequence information, see **Table 92** on page 145 through **Table 98** on page 151.
- For the HiSeq, NextSeq, and NovaSeq platforms, set up the run using the instrument's user interface, following the guidelines on **page 131**.
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed on **page 132** to **page 134** to generate a custom sample sheet.
- Do not use Illumina's IEM adaptor trimming options. Make sure any IEM adaptor trimming option checkboxes are cleared (deselected) when setting up the sequencing run. Adaptors are trimmed in later processing steps using the Agilent software tools described below to ensure proper processing of the degenerate molecular barcodes (MBCs) in the adaptor sequences.
- Demultiplex using Illumina's bcl2fastq, BCL Convert or DRAGEN software to generate paired end reads based on the dual indexes and remove sequences with incorrectly paired P5 and P7 indexes. Do not use the MBC/UMI trimming options in Illumina's demultiplexing software if using Agilent's Genomics NextGen Toolkit (AGeNT), Alissa Reporter, or SureCall software to process your FASTQ files.
- The MBC sequence and dark bases are located at the 5' end of both Read 1 and Read 2. Use the Agilent Genomics NextGen Toolkit (AGeNT) for molecular barcode extraction and trimming (see **page 135** for more information). If your sequence analysis pipeline excludes MBCs and is incompatible with AGeNT, you can trim or mask the first five bases from each read before alignment as described in the *Note* on **page 135**.
- For human germline DNA variant analysis, you can use Agilent's Alissa Reporter software for the complete FASTQ file to variant discovery process (see **page 135** for more information).
- For germline or somatic variant analysis, you can use Agilent's AGeNT software modules to process the library read FASTQ files to analysis-ready BAM files. See **page 136** for more information.
- Library fragments include a degenerate MBC and dark bases at the 5' end of both Read 1 and Read 2 (see **Figure 20** on page 129). If your sequence analysis pipeline excludes MBCs and is incompatible with Alissa Reporter and AGeNT software, you can trim or mask the first five bases from each read before alignment as described in the *Note* on **page 136**.

HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface, using the settings shown in **Table 82**. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface and enter the Cycles settings in **Table 82**.

For the NextSeq or NovaSeq platform, open the *Run Setup* screen of the instrument control software interface and enter the **Read Length** settings in **Table 82**. In the **Custom Primers** section, clear (do **not** select) the check boxes for all primers (*Read 1, Read 2, Index 1* and *Index 2*).

Table 82 Run settings

Run Segment	Cycles/Read Length
Read 1	100 or 150
Index 1 (i7)	8
Index 2 (i5)	8
Read 2	100 or 150

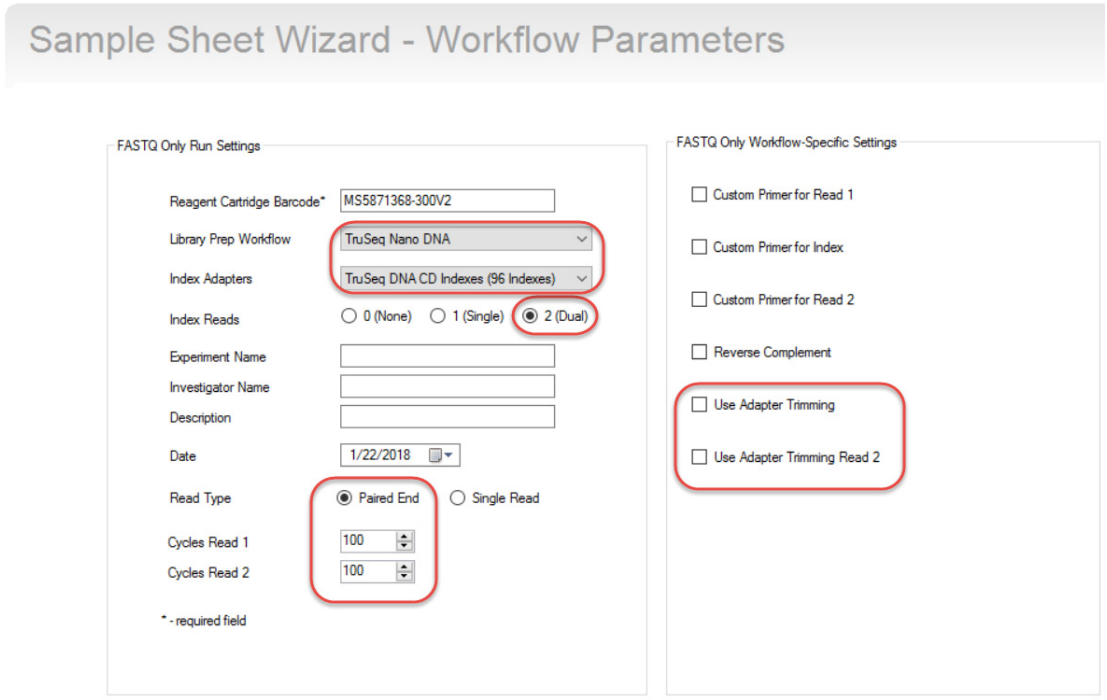
MiSeq platform sequencing run setup guidelines

Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below. Once a Sample Sheet has been generated, index sequences need to be manually changed to the SureSelect XT HS2 indexes used for each sample. See **Table 92** on page 145 through **Table 98** on page 151 for nucleotide sequences of the SureSelect XT HS2 index pairs.

Set up a custom Sample Sheet:

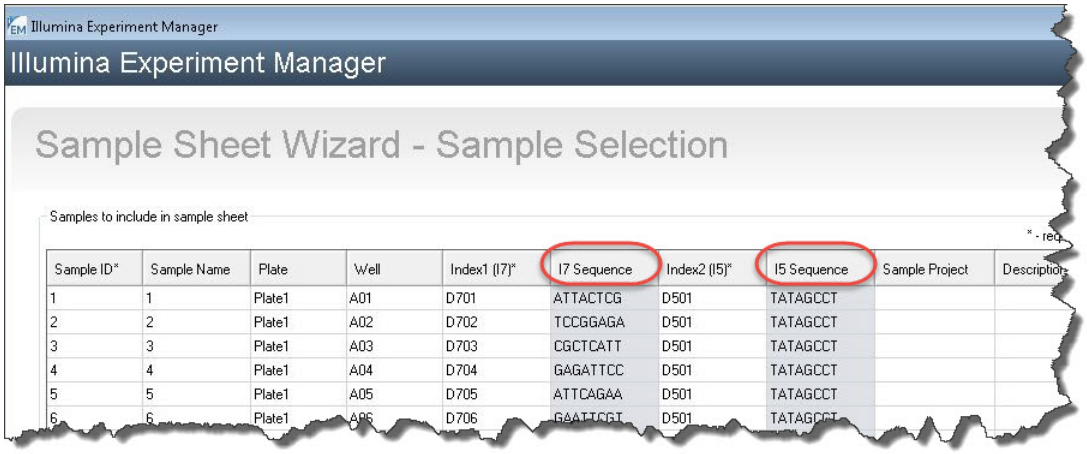
1. In the IEM software, create a Sample Sheet for the MiSeq platform using the following Workflow selections.
   - Under **Category**, select *Other*.
   - Under **Application**, select *FASTQ Only*.
2. On the **Workflow Parameters** screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA.** In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. Clear both adaptor-trimming checkboxes under *FASTQ Only Workflow-Specific Settings*, since adaptor trimming must be performed using Agilent's AGeNT software (see **page 135**).

   If **TruSeq Nano DNA** is not available in the *Sample Prep Kit* field, instead select **TruSeq HT**.



3 Using the **Sample Sheet Wizard**, set up a New Plate, entering the required information for each sample to be sequenced. In the **I7 Sequence** column, assign each sample to any of the Illumina i7 indexes. The index will be corrected to the i7 sequence from the SureSelect XT HS2 index pair at a later stage.

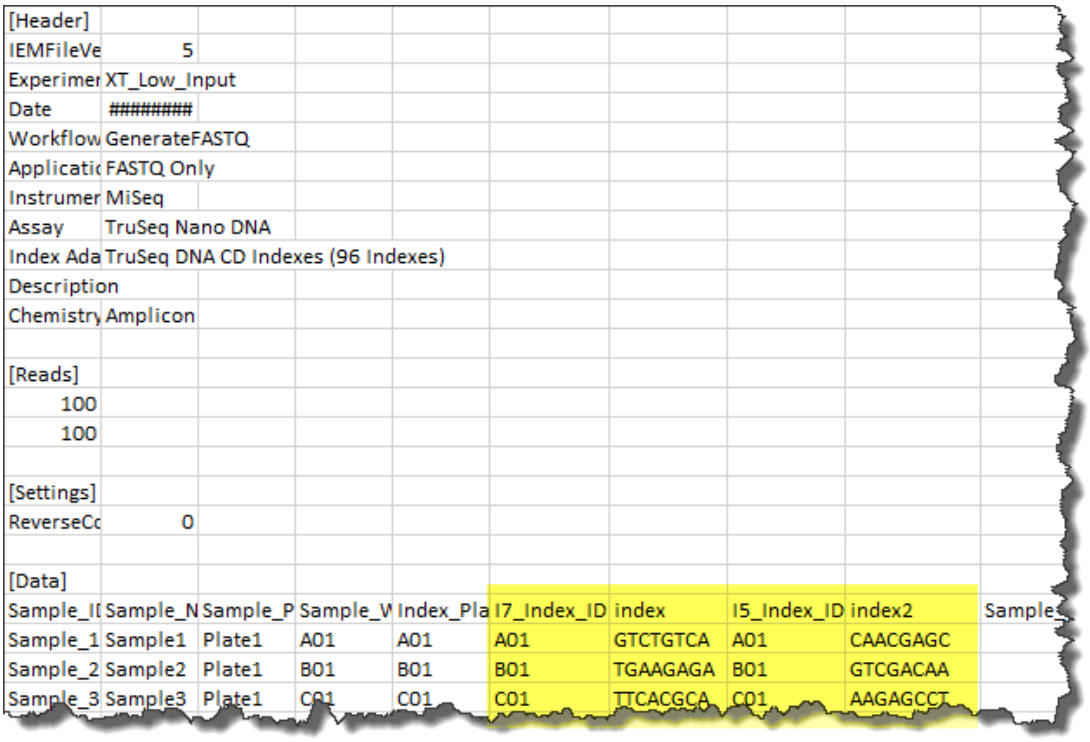
Likewise, in the **I5 Sequence** column, assign any of the Illumina i5 indexes, to be corrected to the i5 sequence from the SureSelect XT HS2 index pair at a later stage.



4 Finish the sample sheet setup tasks and save the sample sheet file.

Editing the Sample Sheet to include SureSelect XT HS2 dual indexes

- Open the Sample Sheet file in a text editor and edit the i7 and i5 index information for each sample in columns 5–8 (highlighted below). See see **Table 92** on page 145 through **Table 98** on page 151 for nucleotide sequences of the SureSelect XT HS2 indexes.
- In column 5 under **I7_Index_ID**, enter the SureSelect XT HS2 index pair number assigned to the sample. In column 6 under **index**, enter the corresponding P7 index sequence.
- In column 7 under **I5_Index_ID**, enter the SureSelect XT HS2 index pair number assigned to the sample. In column 8 under **index2**, enter the corresponding P5 index sequence.
- If the run includes more than 96 samples, the sample sheet may be edited to include additional sample rows containing the assigned SureSelect XT HS2 index pair sequences in column 6 (P7 index) and column 8 (P5 index).



[Header]									
IEMFileVe	5								
Experimer	XT_Low_Input								
Date	########								
Workflow	GenerateFASTQ								
Applicatic	FASTQ Only								
Instrumer	MiSeq								
Assay	TruSeq Nano DNA								
Index Ada	TruSeq DNA CD Indexes (96 Indexes)								
Description									
Chemistry	Amplicon								
[Reads]									
100									
100									
[Settings]									
ReverseCc	0								
[Data]									
Sample_ID	Sample_N	Sample_P	Sample_W	Index_Pla	I7_Index_ID	index	I5_Index_ID	index2	Sample
Sample_1	Sample1	Plate1	A01	A01	A01	GTCTGTCA	A01	CAACGAGC	
Sample_2	Sample2	Plate1	B01	B01	B01	TGAAGAGA	B01	GTCGACAA	
Sample_3	Sample3	Plate1	C01	C01	C01	TTCACGCA	C01	AAGAGCCT	

Figure 21 Sample sheet for SureSelect XT HS2 library sequencing

5 Save the edited Sample Sheet in an appropriate file location for use in the run.

Sequence analysis resources

Guidelines are provided below for typical NGS analysis pipeline steps appropriate for SureSelect XT HS2 DNA library data analysis. Your NGS analysis pipeline may vary.

Use Illumina's bcl2fastq, BCL Convert or DRAGEN software to generate paired end reads by demultiplexing sequences based on the dual indexes. The demultiplexed FASTQ data needs to be pre-processed to remove sequencing adaptors and extract the MBC sequences using one of the tools described below.

Using Agilent's Alissa Reporter software for germline DNA workflows

Alissa Reporter software provides a complete FASTQ-to-Report solution for Agilent's SureSelect assays, processing NGS data from FASTQ format to VCF format, and reporting human germline SNV, InDel and CNV calls.

Alissa Reporter is a cloud-based, multi-tenant software as a service (SaaS) product, delivering integrated pre-processing of SureSelect XT HS2 DNA library reads (adaptor trimming, MBC extraction and de-duplication) along with secondary data analysis and quality control (QC) analytics using a built-in dashboard. To obtain more information and to purchase access to the software please visit the Alissa Reporter page at www.agilent.com.

Key considerations for SureSelect XT HS2 DNA assay steps prior to Alissa Reporter software analysis are summarized below:

- Alissa Reporter applications are available for germline analysis of human DNA libraries enriched using a pre-designed or custom SureSelect human probe (see **page 15**). Libraries enriched using SureSelect XT HS Human All Exon V7 or V8 probes are analyzed with the corresponding *Human All Exon V7 Germline* or *Human All Exon V8 Germline* application in Alissa Reporter. Libraries enriched using other probes, including additional pre-designed probes, are analyzed using an Alissa Reporter *Custom* application. The Alissa Reporter console provides tools for importing both pre-designed probe and custom probe designs from SureDesign and setting up a new *Custom* application for each imported design.

NOTE

Human All Exon V8+UTR and Human All Exon V8+NCV designs must be imported into Alissa Reporter for use as *Custom*-type applications. Use the *Catalog*-type Alissa Reporter applications, including the *Human All Exon V8 Germline* application, only for the specific probe indicated for the application without any additional design content.

- Analysis of FFPE-derived or other DNA samples for detection of somatic variants is not supported at the time of this publication.
- For CNV calling a co-analysis strategy is used in which unrelated samples from the same Alissa Reporter run are used to determine the reference signal for the target sample (no specific reference sample is required). At least 3 and preferably 8 (or more) unrelated samples need to be analyzed in Alissa Reporter together to obtain a reliable reference signal for CNV calls. For CNV calling on the X and Y chromosomes, unrelated samples of the same gender are required. For best results, process the samples to be used for CNV co-analysis in the same SureSelect run and in the same sequencing run in order to minimize any processing-based variance.
- Maximum file size for uploads is 50GB/file (in total 400GB/sample). A maximum of 768 FASTQ files can be uploaded in a run.

- File sizes >150M reads are randomly subsampled to 150M reads when using the *Human All Exon V7 Germline* or *Human All Exon V8 Germline* application.
- Unmerged and merged FASTQ files are supported. Upload of BAM files or other non-FASTQ file formats is not supported.

Using Agilent's AGeNT software for germline or somatic DNA workflows

Agilent's AGeNT software is a Java-based toolkit used for SureSelect XT HS2 DNA library read processing steps. The AGeNT tools are designed to enable building, integrating, maintaining, and troubleshooting internal analysis pipelines for users with bioinformatics expertise. For additional information and to download this toolkit, visit the AGeNT page at www.agilent.com and review the AGeNT Best Practices document for processing steps suitable for XT HS2 DNA libraries.

Prior to variant discovery, demultiplexed SureSelect XT HS2 library FASTQ data are pre-processed to remove sequencing adaptors and extract the MBC sequences using the AGeNT Trimmer module.

The trimmed reads should be aligned, and MBC tags added to the aligned BAM file using a suitable tool such as BWA-MEM. Once alignment and tagging are complete, the AGeNT CReaK (Consensus Read Kit) tool is used to generate consensus reads and mark or remove duplicates. The resulting BAM files are ready for downstream analysis including variant discovery.

NOTE

CReaK is a deduplication tool introduced in AGeNT version 3.0, replacing the AGeNT LocatIt tool. Please visit the AGeNT page at www.agilent.com and review the FAQs for a detailed comparison of LocatIt and CReaK. LocatIt remains available for backward compatibility but CReaK is the recommended tool.

NOTE

If 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 * may be replaced with the actual read length, matching 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 N5Y145;I8;I8;N5Y145 for 2x150 NGS).

Alternatively, the first 5 bases may be trimmed from the demultiplexed fastq files using a suitable processing tool of your choice, such as seqtk. The AGeNT Trimmer module can also be used to remove the MBCs while trimming adaptor sequences. Standard adaptor trimmers will fail to remove the MBC sequences from the opposite adaptor (refer to **Figure 20**), which may affect alignment quality.

7 Appendix: Using FFPE-derived DNA Samples

This chapter summarizes the protocol modifications to apply to FFPE samples based on the integrity of the FFPE sample DNA.

Protocol modifications for FFPE Samples

Protocol modifications that should be applied to FFPE samples are summarized in **Table 83**.

Table 83 Summary of protocol modifications for FFPE samples

Workflow Step and page	Parameter	Condition for non-FFPE Samples	Condition for FFPE Samples
gDNA Sample Preparation **page 46**	Qualification of DNA Integrity	Not required	Required
Enzymatic fragmentation duration **page 49**	Duration of the 37°C fragmentation step	15–25 minutes, depending on read length requirements	25 minutes
DNA input for Library Preparation **page 47**	Input amount and means of quantification	10 ng to 200 ng, quantified by Qubit assay	Based on determined DNA integrity (see **Table 17** on page 47 and **Table 18** on page 47)
DNA Shearing **page 55**	Mode of DNA Shearing	2 × 120 seconds	240 seconds (continuous)
Pre-capture PCR **page 64**	Cycle number	8–11	11–14
Sequencing **page 140**	Output augmentation	Per project requirements	1× to 10× based on determined DNA integrity (see **Table 84** and **Table 85** on page 140)

Methods for FFPE Sample Qualification

DNA integrity may be assessed using the Agilent NGS FFPE QC Kit or using the Agilent 4200 TapeStation system and Genomic DNA ScreenTape.

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include the precise quantity of amplifiable DNA in the sample to allow direct normalization of input DNA amount and a ΔΔCq DNA integrity score used to design other protocol modifications.

The Agilent 4200 TapeStation system, combined with the Genomic DNA ScreenTape assay, provides a electrophoresis-based method for determination of a DNA Integrity Number (DIN) score used to estimate amount of input DNA required for sample normalization and to design other protocol modifications.

Sequencing Output Recommendations for FFPE Samples

After determining the amount of sequencing output required for intact DNA samples to meet the goals of your project, use the guidelines below to determine the amount of extra sequencing output required for FFPE DNA samples.

Samples qualified using ΔΔCq For samples qualified based on the ΔΔCq DNA integrity score, use the guidelines in **Table 84**. For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with ΔΔCq score of 1 requires 200–400 Mb of sequencing output to achieve the same coverage.

Table 84 Recommended sequencing augmentation for FFPE-derived DNA samples

ΔΔCq value	Recommended fold increase for FFPE-derived sample
<0.5	No extra sequencing output
between 0.5 and 2	Increase sequencing allocation by 2× to 4×
>2	Increase sequencing allocation by 5× to 10× or more

Samples qualified using DIN For samples qualified based on the Genomic DNA ScreenTape assay DIN integrity score, use the guidelines in **Table 85**. For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with DIN score of 4 requires approximately 200–400 Mb of sequencing output to achieve the same coverage.

Table 85 Recommended sequencing augmentation for FFPE-derived DNA samples

DIN value	Recommended fold increase for FFPE-derived sample
≥8	No extra sequencing output
between 3 and 8	Increase sequencing allocation by 2× to 4×
<3	Increase sequencing allocation by 5× to 10× or more

8 Reference

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

Kit Contents

The SureSelect XT HS2 DNA System protocol using the Agilent NGS Bravo uses the kits listed in **Table 86**. Detailed contents of each of the multi-part component kits listed in **Table 86** are shown in **Table 87** through **Table 90** on the following pages.

Table 86 Component Kits

Kit Name (p/n)	Component Kit Name	Component Kit p/n	Storage Condition
SureSelect XT HS2 DNA Library Preparation Kit for ILM (Pre PCR), 96 Reactions (G9985A through G9985D)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR)	5500-0147	–20°C
	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	5191-5688 (Index Pairs 1–96), 5191-5689 (Index Pairs 97–192), 5191-5690 (Index Pairs 193–288), OR 5191-5691 (Index Pairs 289–384)	–20°C
SureSelect XT HS2 DNA Target Enrichment Kit (Post PCR), 12 Hybs (G9987A)	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	5191-6689	Room Temperature
	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	5191-6690	–20°C
SureSelect XT HS2 DNA Reagent Kit, 96 Reactions (G9983A through G9983D; or G9984A through G9984D with AMPure XP/ Streptavidin Beads)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR)	5500-0147	–20°C
	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	5191-5688 through 5191-5691	–20°C
	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	5190-9687	Room Temperature
	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	5191-6688	–20°C
	SureSelect DNA AMPure XP Beads (included with kits G9984A through G9984D)	5191-5740	+4°C
	SureSelect Streptavidin Beads (included with kits G9984A through G9984D)	5191-5742	+4°C
SureSelect Enzymatic Fragmentation Kit, 96 Reactions Automation (5191-6764)			–20°C

Table 87 SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR) Content

Kit Component	Format
End Repair-A Tailing Enzyme Mix	tube with orange cap
End Repair-A Tailing Buffer	bottle
T4 DNA Ligase	tube with blue cap
Ligation Buffer	bottle
SureSelect XT HS2 Adaptor Oligo Mix	tube with white cap
Herculase II Fusion DNA Polymerase	tube with red cap
5× Herculase II Reaction Buffer with dNTPs	tube with clear cap

Table 88 SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) Content

Kit Component	Format
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	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)

Table 89 SureSelect Target Enrichment Kit, ILM Hyb Module Box 1 (Post PCR) Content

Kit Component	Format
SureSelect Binding Buffer	bottle
SureSelect Wash Buffer 1	bottle
SureSelect Wash Buffer 2	bottle

Table 90 SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR) Content

Kit Component	Format
SureSelect Fast Hybridization Buffer	bottle
SureSelect XT HS2 Blocker Mix	tube with blue cap
SureSelect RNase Block	tube with purple cap
SureSelect Post-Capture Primer Mix	tube with clear cap
Herculase II Fusion DNA Polymerase	tube with red cap
5× Herculase II Reaction Buffer with dNTPs	tube with clear cap

SureSelect XT HS2 Index Primer Pair Information

The SureSelect XT HS2 Index Primer Pairs are provided pre-combined. Each member of the primer pair contains a unique 8-bp P7 or P5 index, resulting in dual-indexed NGS libraries. One primer pair is provided in each well of 96-well plates. Each well contains a single-use aliquot of a specific pair of P7 plus P5 primers.

The nucleotide sequence of the index portion of each primer is provided in **Table 92** through **Table 98**. 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. Illumina sequencing platforms and their P5 sequencing orientation are shown in **Table 91**. 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 91 P5 index sequencing orientation by Illumina platform

P5 Index Orientation	Platform
Forward	NovaSeq 6000 with v1.0 chemistry MiSeq HiSeq 2500
Reverse Complement*	NovaSeq 6000 with v1.5 chemistry NextSeq 500/550/1000/2000 HiSeq 3000/4000 iSeq 100 MiniSeq HiSeq X

* 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.

Table 92 SureSelect XT HS2 Index Primer Pairs 1–48, provided in orange 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
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 93 SureSelect XT HS2 Index Primer Pairs 49–96, provided in orange 96-well plate

Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement	Primer Pair #	Well	P7 Index Forward	P5 Index Forward	P5 Index Reverse Complement
49	A07	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 94 SureSelect XT HS2 Index Primer Pairs 97–144, provided in blue 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
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 95 SureSelect XT HS2 Index Primer Pairs 145–192, provided in blue 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
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 96 SureSelect XT HS2 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 97 SureSelect XT HS2 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 98 SureSelect XT HS2 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

Index Primer Pair Plate Maps

Table 99 Plate map for SureSelect XT HS2 Index Primer Pairs 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 100 Plate map for SureSelect XT HS2 Index Primer Pairs 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	1110	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 101 Plate map for SureSelect XT HS2 Index Primer Pairs 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 102 Plate map for SureSelect XT HS2 Index Primer Pairs 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 XT HS2 DNA System Protocol using NGS Bravo Option A protocol.

Enzymatic Fragmentation

Table 103 Fragmentation master mix - used on page 50

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	2 µL	42.5	59.5	76.5	97.8	119.0	136.0	153.0	170.0	191.3	208.3	225.3	253.8
5X SureSelect Fragmentation Buffer (blue cap)	2 µL	42.5	59.5	76.5	97.8	119.0	136.0	153.0	170.0	191.3	208.3	225.3	253.8
SureSelect Fragmentation Enzyme (green cap)	1 µL	21.3	29.8	38.3	48.9	59.5	68.0	76.5	85.0	95.6	104.1	112.6	126.9
Total Volume	**5 µL**	**106.3**	**148.8**	**191.3**	**244.5**	**297.5**	**340.0**	**382.5**	**425.0**	**478.2**	**620.7**	**583.2**	**634.4**

Table 104 Master mix source plate for 00a EnzFrag_XT_HS2_ILM protocol - used on page 50

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Selected Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Fragmentation master mix	Column 1 (A1-H1)	12.5	17.5	22.5	28.8	35.0	40.0	45.8	51.5	57.3	63.0	68.8	75.0

Library Preparation

Table 105 End Repair/dA-Tailing master mix - used on page 57

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
End Repair-A Tailing Buffer (yellow cap or bottle)	16 µL	204.0	340.0	476.0	612.0	748.0	884.0	1042.7	1201.3	1360.0	1518.7	1677.3	1836.0
End Repair-A Tailing Enzyme Mix (orange cap)	4 µL	51.0	85.0	119.0	153.0	187.0	221.0	260.7	300.3	340.0	379.7	419.3	459.0
Total Volume	**20 µL**	**255.0**	**425.0**	**595.0**	**765.0**	**935.0**	**1105.0**	**1303.4**	**1501.6**	**1700.0**	**1898.4**	**2096.6**	**2295.0**

Table 106 Ligation master mix - used on page 58

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Ligation Buffer (purple cap or bottle)	23 µL	293.3	488.8	684.3	879.8	1075.3	1270.8	1515.1	1759.5	2003.9	2248.3	2492.6	2737.0
T4 DNA Ligase (blue cap)	2 µL	25.5	42.5	59.5	76.5	93.5	110.5	127.5	153.0	174.3	195.5	216.8	238.0
Total Volume	**25 µL**	**318.8**	**531.3**	**743.8**	**956.3**	**1168.8**	**1381.3**	**1642.6**	**1912.5**	**2178.2**	**2443.8**	**2709.4**	**2975.0**

Table 107 Adaptor Oligo Mix dilution - used on page 58

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	2.5 µL	42.5	63.8	85.0	106.3	127.5	148.8	170.0	191.3	212.5	233.8	255.0	276.3
SureSelect XT HS2 Adaptor Oligo Mix (white cap)	5 µL	85.0	127.5	170.0	212.5	255.0	297.5	340.0	382.5	425.0	467.5	510.0	552.5
Total Volume	**7.5 µL**	**127.5**	**191.3**	**255.0**	**318.8**	**382.5**	**446.3**	**510.0**	**573.8**	**637.5**	**701.3**	**765.0**	**828.8**

Table 108 Master mix source plate for 01 LibraryPrep_XT_HS2_ILM protocol - used on page 59

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Agilent Deep Well Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
End Repair-dA Tailing master mix	Column 1 (A1-H1)	31.0	52.0	73.0	94.0	115.0	136.0	158.8	182.0	205.8	230.0	254.8	280.0
Ligation master mix	Column 2 (A2-H2)	36.0	62.0	88.0	114.0	140.0	166.0	195.7	226.5	258.3	291.1	325.1	360.0
Adaptor Oligo Mix dilution	Column 3 (A3-H3)	15.0	22.5	30.0	37.5	45.0	52.5	60.6	68.8	76.9	85.0	93.1	101.3

Pre-Capture PCR

Table 109 Pre-Capture PCR Master Mix - used on page 65

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
5× Herculase II Buffer with dNTPs (clear cap)	10 µL	170	255	340	425	510.0	574	656.0	738.0	820.0	902.0	984.0	1066
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17	25.5	34	42.5	51.0	57.4	65.6	73.8	82.0	90.2	98.4	106.6
Total Volume	**11 µL**	**187**	**280.5**	**374**	**467.5**	**561.0**	**631.4**	**721.6**	**811.8**	**902.0**	**992.2**	**1082.4**	**1172.6**

Table 110 Master mix source plate for 03 Pre-CapPCR_XT_HS2_ILM protocol - used on page 65

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Selected Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Pre-Capture PCR Master Mix	Column 2 (A2-H2)	22	33	44	55	66	77	88	99	110	121	132	143

Hybridization

Table 111 Block master mix - used on page 95

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	2.5 µL	31.9	53.1	74.4	95.6	116.9	138.1	161.1	184.2	207.2	230.2	253.2	276.3
SureSelect XT HS2 Blocker Mix (blue cap)	5.0 µL	63.8	106.3	148.8	191.3	233.8	276.3	322.3	368.3	414.4	460.4	506.5	552.5
Total Volume	**7.5 µL**	**95.7**	**159.4**	**223.2**	**286.9**	**350.7**	**414.4**	**483.4**	**552.5**	**621.6**	**690.6**	**759.7**	**828.8**

Table 112 Probe Hybridization master mix for Probes <3 Mb, 8 rows of wells - used on page 96

Reagent	Volume for 1 Library	Target size <3.0 Mb Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	7.0 µL	89.3	148.8	208.3	267.8	327.3	401.6	471.0	540.5	609.9	679.3	748.7	818.1
RNase Block (purple cap)	0.5 µL	6.4	10.6	14.9	19.1	23.4	28.7	33.6	38.6	43.6	48.5	53.5	58.4
SureSelect Fast Hybridization Buffer	6.0 µL	76.5	127.5	178.5	229.5	280.5	344.3	403.8	463.3	522.8	582.3	641.8	701.3
Probe (with design <3.0 Mb)	2.0 µL	25.5	42.5	59.5	76.5	93.5	114.8	134.6	154.4	174.3	194.1	213.9	233.8
Total Volume	**15.5 µL**	**197.7**	**329.4**	**461.2**	**592.9**	**724.7**	**889.4**	**1043.0**	**1196.8**	**1350.6**	**1504.2**	**1657.9**	**1811.6**

Table 113 Probe Hybridization master mix for Probes ≥3 Mb, 8 rows of wells - used on page 96

Reagent	Volume for 1 Library	Target size ≥3.0 Mb Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	4.0 µL	51.0	85.0	119.0	153.0	187.0	229.5	269.2	308.8	348.5	388.2	427.8	467.5
RNase Block (purple cap)	0.5 µL	6.4	10.6	14.9	19.1	23.4	28.7	33.6	38.6	43.6	48.5	53.5	58.4
SureSelect Fast Hybridization Buffer	6 µL	76.5	127.5	178.5	229.5	280.5	344.3	403.8	463.3	522.8	582.3	641.8	701.3
Probe (with design ≥3.0 Mb)	5.0 µL	63.8	106.3	148.8	191.3	233.8	286.9	336.5	386.0	435.6	485.2	534.8	584.4
Total Volume	**15.5 µL**	**197.7**	**329.4**	**461.2**	**592.9**	**724.7**	**889.4**	**1043.1**	**1196.7**	**1350.5**	**1504.2**	**1657.9**	**1811.6**

Table 114 Probe Hybridization master mix for Probes <3 Mb, single row of wells - used on page 97

Reagent	Volume for 1 Library	Target size <3.0 Mb Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	7	10.5	17.5	24.5	31.5	38.5	49.0	57.2	65.3	73.5	81.7	89.8	98.0
RNase Block (purple cap)	0.5	0.8	1.3	1.8	2.3	2.8	3.5	4.1	4.7	5.3	5.8	6.4	7.0
SureSelect Fast Hybridization Buffer	6.0	9.0	15.0	21.0	27.0	33.0	42.0	49.0	56.0	63.0	70.0	77.0	84.0
Probe (with design <3 Mb)	2.0	3.0	5.0	7.0	9.0	11.0	14.0	16.3	18.7	21.0	23.3	25.7	28.0
Total Volume	**15.5**	**23.3**	**38.8**	**54.3**	**69.8**	**85.3**	**108.5**	**126.6**	**144.7**	**162.8**	**180.8**	**198.9**	**217.0**

Table 115 Probe Hybridization master mix for Probes ≥3 Mb, single row of wells - used on page 97

Reagent	Volume for 1 Library	Target size ≥3.0 Mb Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	4.0	6.0	10.0	14.0	18.0	22.0	28.0	32.7	37.3	42.0	46.7	51.3	56.0
RNase Block (purple cap)	0.5	0.8	1.3	1.8	2.3	2.8	3.5	4.1	4.7	5.3	5.8	6.4	7.0
SureSelect Fast Hybridization Buffer	6.0	9.0	15.0	21.0	27.0	33.0	42.0	49.9	56.0	63.0	70.0	77.0	84.0
Probe (with design ≥3 Mb)	5.0	7.5	12.5	17.5	22.5	27.5	35.0	40.8	46.7	52.5	58.3	64.2	70.0
Total Volume	**15.5**	**23.3**	**38.8**	**54.3**	**69.8**	**85.3**	**108.5**	**126.6**	**144.7**	**162.8**	**180.8**	**198.9**	**217.0**

Table 116 Master mix source plate for 07 Hyb_XT_HS2_ILM protocol - used on page 98

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Block master mix	Column 1 (A1-H1)	11.0	19.0	27.0	34.9	42.9	50.9	59.5	68.1	76.8	85.4	94.0	102.7
Probe Hybridization master mix	Column 2 (A2-H2)	23.3	38.8	54.3	69.8	85.3	108.5	126.6	144.7	162.8	180.8	198.9	217.0*

* Wells containing 217.0 µL are nearly full. Pipette carefully to avoid introducing bubbles to the bottom of the wells. Handle the plate with care to avoid spillage. Do not centrifuge.

Hybrid Capture and Washing

Table 117 Magnetic bead washing mixture - used on page 104

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Dynabeads MyOne Streptavidin T1 bead suspension	50 µL	425	825	1225	1650	2050	2500	2900	3350	3750	4200	4600	5000
SureSelect Binding Buffer	200 µL	1700	3300	4900	6600	8200	10000	11600	13400	15000	16800	18400	20000
Total Volume	**250 µL**	**2125**	**4125**	**6125**	**8250**	**10260**	**12500**	**14500**	**16750**	**18750**	**21000**	**23000**	**25000**

Table 118 Resuspension volumes for washed bead suspension - used on page 104

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
SureSelect Binding Buffer	200 µL	1700	3300	4900	6600	8200	10000	11600	13400	15000	16800	18400	20000

Post-Capture PCR

Table 119 Post-Capture PCR master mix - used on page 113

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
5× Herculase II Reaction Buffer with dNTPs (clear cap)	10 µL	170	255	340	425	510	595	683.3	772.5	862.5	953.3	1045	1137.5
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	17.0	25.5	34.0	42.5	51.0	59.5	68.3	77.3	86.3	95.3	104.5	113.8
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17.0	25.5	34.0	42.5	51.0	59.5	68.3	77.3	86.3	95.3	104.5	113.8
Total Volume	**12.0 µL**	**204.0**	**306.0**	**408.0**	**510.0**	**612.0**	**714.0**	**819.9**	**927.1**	**1035.1**	**1143.9**	**1254.0**	**1365.1**

Table 120 Master mix source plate for 10 Post-CapPCR_XT_HS2_ILM protocol - used on page 114

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Selected Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Post-Capture PCR Master Mix	Column 3 (A3-H3)	23.0	36.0	49.0	62.0	75.0	88.0	101.0	114.0	127.0	140.0	153.0	166.0

Quick Reference Tables for Other Reagent Volumes

This section contains tables that summarize the gDNA input volumes, volume of XT HS2 Index Primer Pair in the primer plate, volumes used for reservoirs of water and ethanol, and volumes of AMPure XP beads used in the automation protocols.

Table 121 Genomic DNA Input Volumes

Genomic DNA Input (100–200 ng)	Volume for 1 Library
Enzymatic fragmentation	15 µL
Mechanical shearing	50 µL

Table 122 XT HS2 Index Primer Pairs Volume on Primer Plate

Reagent	Volume for 1 Library
XT HS2 Index Primer Pairs	5 µL

Table 123 AMPure XP Bead Volumes for AMPure XP Protocols

Protocol or Runset	Volume of AMPure Beads per Well*
LibraryPrep_XT_HS2_ILM	80 µL
AMPureXP_XT_HS2_ILM (Pre-Cap PCR - SinglePlex)	50 µL
AMPureXP_XT_HS2_ILM (Pre-Cap PCR - MultiPlex)	50 µL
AMPureXP_XT_HS2_ILM (Concentration of Pool)	180 µL
AMPureXP_XT_HS2_ILM (Post-Capture PCR)	50 µL

* When preparing the plates of AMPure XP beads, fill the columns of the reservoir with enough of the bead suspension to cover the pyramid-shaped wells

Table 124 Water and Ethanol Volumes for AMPure XP 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

- ✔ The library preparation protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance of the highly viscous buffer and enzyme solutions used in the protocol. Be sure to adhere to all instructions when setting up the reactions.
- ✔ PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the 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 overamplified. Repeat library preparation for the sample, decreasing the pre-capture PCR cycle number by 1 to 3 cycles.
- ✔ DNA isolated from degraded samples, including 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.
- ✔ Performance of the solid-phase reversible immobilization (SPRI) purification step may be poor. Verify the expiration date for the vial of AMPure XP beads used for purification. 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 SPRI procedure.

If solids observed in the End Repair-A Tailing Buffer

- ✔ Vortex the solution at high speed until the solids are dissolved. The observation of solids when first thawed does not impact performance, but it is important to mix the buffer until all solutes are dissolved.

If pre-capture library fragment size is larger than expected in electropherograms

- ✔ Shearing may not be optimal. For intact, high-quality DNA samples, ensure that mechanical shearing is completed using the two-round shearing protocol provided, including all spinning and vortexing steps.
- ✔ When using the mechanical shearing method, any bubbles present on the microTUBE filament may disrupt complete shearing. Spin the microTUBE for 30 seconds before the first round of shearing to ensure that any bubbles are released.

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 target DNA shear size.
- ✔ DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. When preparing the AMPure XP bead plate for pre-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 AMPure XP bead plate, seal the plate and store at 4°C until needed.

If low molecular weight adaptor-dimer peak is present in pre-capture library electropherograms

- ✔ The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to the samples analyzed on **page 75**. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield of pre-capture libraries.
- ✔ For whole-genome sequencing (not specifically supported by this protocol), samples with an adaptor-dimer peak must be subjected to an additional round of SPRI-purification. To complete, first dilute the sample to 50 µL with nuclease free water, then run the AMPureXP_XT_HS2_ILM (Pre-Capture PCR) protocol.

If yield of post-capture libraries is low

- ✔ 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.
- ✔ The RNA 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 kept on ice until it is dispensed into the master mix source plate, as directed on **page 96**, and that solutions containing the Probe are not held at room temperature for extended periods.

If post-capture library fragment size is different than expected in electropherograms

- ✔ DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. When preparing the AMPure XP bead 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 AMPure XP 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 Bravo NGS Bravo to retain the 65°C sample temperature during transfer step (**step 27** on **page 102**).

If low uniformity of coverage with high AT-dropout is observed in library sequencing results

✔ High AT-dropout may indicate that hybridization conditions are too stringent to obtain the desired level of coverage for AT-rich targets.

- For libraries target-enriched using the SureSelect XT HS Human All Exon V8 Probe or SureSelect XT HS PreCap Human All Exon V8 Probe and the hybridization program in **Table 52** on page 94 (including segment with one-hour incubation at 65°C), repeat target enrichment using the hybridization program in **Table 53** on page 94 (without the one-hour incubation at 65°C segment).
- For all other probes, repeat target enrichment at lower stringency using a modified thermal cycler program for hybridization, reducing the hybridization temperature in segments 4 and 5 from 65°C to 62.5°C or 60°C (see **Table 53** on page 94).

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In This Book

This guide contains information to run the SureSelect XT HS2 protocol for DNA, with optional pre-capture pooling, using automation protocols provided with the Agilent NGS Bravo Option A.

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