SureSelect XT HS2 DNA Kits

Automated using Agilent Bravo NGS Workstation Option B+ with an on-deck thermal cycler (ODTC)

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

Protocol

Version A0, August 2023

SureSelect platform manufactured with Agilent SurePrint technology.

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

This protocol applies only to the Bravo NGS Workstation Option B+, which includes an on-deck thermal cycler on the Bravo deck. Make sure this protocol is the correct one for your system type before proceeding.

Notices

Manual Part Number

G9985-90015

Edition

Version A0, August 2023

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Acknowledgment

Technical Support

For US and Canada

Call (800) 227-9770 (option 3,4,4)

Or send an e-mail to:
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For all other regions

Agilent's world-wide Sales and Support Center contact details for your location can be obtained at
www.agilent.com/en/contact-us/page.

Warranty

Safety Notices

CAUTION

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

WARNING

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

In this Guide...

This guide provides an optimized protocol for preparation of target-enriched Illumina paired-end multiplexed sequencing libraries using SureSelect XT HS2 Reagent Kits. Sample processing steps are automated using the Agilent Bravo NGS Workstation Option B+, featuring an on-deck thermal cycler.

1 Before You Begin

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

2 Overview of the Agilent Bravo NGS Workstation Option B+

This chapter contains an orientation to the Agilent Bravo NGS Workstation Option B+ and considerations for designing SureSelect experiments for automated processing using the NGS Workstation Option B+.

3 Overview of SureSelect XT HS2 DNA Automation

This chapter contains an overview of the SureSelect XT HS2 DNA target enrichment workflow and the SureSelect XT HS2 DNA VWorks form for the NGS Workstation Option B+.

4 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 VWorks Utility form.

5 DNA Fragmentation and Library Preparation

This chapter provides instructions for DNA fragmentation and DNA library preparation for the Illumina paired-read sequencing platform. For each sample to be sequenced, an individual indexed and molecular-barcoded library is prepared.

6 Target Enrichment

This chapter provides instructions to complete the target enrichment steps, including hybridization and capture using a SureSelect Probe.

7 Sequencing Preparation and Processing

This chapter provides instructions for preparing samples for sequencing, guidelines for performing the sequencing, and information on sequence data analysis.

8 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.

9 Reference

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

Content

1 Before You Begin

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

Make sure you have the most current protocol. Go to **www.agilent.com** and search for G9985-90015.

NOTE

This Protocol describes automated DNA sample processing for SureSelect XT HS2 Target Enrichment using the Agilent Bravo NGS Workstation Option B+. If you are not using Option B+, which features an on-deck thermal cycler, you need to use a different Protocol. Publications can be downloaded from **www.agilent.com**. See publication numbers below.

- SureSelect XT HS2 DNA System automated for **Bravo NGS Option A**: See publication G9985-90020
- SureSelect XT HS2 DNA System automated for **Bravo NGS Workstation Option B**: See publication G9985-90010

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

- Clear the NGS Workstation of all plates and tip boxes prior to setting up an automated protocol or runset.
- Load the NGS Workstation as detailed in each of the protocol steps before initiating each automated protocol run. When loading plates in the workstation's Labware MiniHub, always place plates in the orientation shown in **Figure 8** on page 61.
- Many of the protocols and runsets require an Agilent shallow well reservoir containing nuclease-free water and/or an Agilent deep well reservoir containing 70% ethanol. You can reuse these reservoirs in multiple protocols and runsets that are being performed in the same day. Do not reuse reservoirs that were used a previous day or with a different reagent. Use fresh nuclease-free water and freshly prepared 70% ethanol every time.
- To prevent evaporation during setup of a run, add a lid to reservoirs containing 70% ethanol.
- Some workflow steps require that the sample plate be sealed using the PlateLoc thermal microplate sealer included with the NGS Workstation, and then centrifuged to collect any dispersed liquid. To maximize efficiency, locate the plate centrifuge in close proximity to the NGS Workstation.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Use best-practices to prevent PCR product contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR work areas and use dedicated supplies and reagents in each area. In particular, never use materials designated to post-PCR work for pre-PCR segments of the workflow. Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
  2. Maintain clean work areas. Clean pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution.
  3. Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.
- In general, follow Biosafety Level 1 (BSL1) safety rules.
- Possible stopping points, where samples may be stored at –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.

Safety Notes

WARNING

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

Ethanol is flammable. When working with ethanol, keep away from open flames. Consult the safety data sheet provided by the ethanol vendor for information on waste disposal.

For Agilent reagent safety information, consult the safety data sheets and any product hazard labels. Agilent safety data sheets are available at www.agilent.com.

Materials Required

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

- DNA sample type: high-quality genomic DNA (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.

- **Table 1**: Equipment and reagents for all sample types and workflows.
- **Table 2**: Agilent Bravo NGS Workstation and required plasticware for the NGS Workstation.
- **Table 3**: SureSelect XT HS2 DNA Reagent Kits suitable for both pre-capture pooling workflows and post-capture pooling workflows.
- **Table 4**: Compatible Probes suitable for both pre-capture pooling workflows and post-capture pooling workflows.
- **Table 5**: Additional materials needed based on DNA sample type and fragmentation method.
- **Table 6**: Equipment and reagents needed based on your selected nucleic acid analysis platform.
- **Table 7**: Supplies for decontaminating the NGS Workstation.

Table 1 Required Equipment and 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
Tween 20 – Needed if you store the library before sequencing	Sigma-Aldrich p/n P9416-50ML
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

Table 1 Required Equipment and Reagents -- All Sample and Workflow Types (continued)

Description	Vendor and Part Number
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
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

* 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 Bravo NGS Workstation and Workstation Plasticware -- All Sample and Workflow Types

Description	Vendor and Part Number
Agilent Bravo NGS Workstation Option B+ Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5574AA with options 006 and 008 (VWorks software version 14.2.0.1534) OR Agilent p/n G5574AA or G5522A, upgraded with Agilent p/n G5297GA (VWorks software version 14.2.0.1534)
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Processing plates, 96-wells, full-skirted	96-well Eppendorf twin.tec plates (Eppendorf p/n 951020401) OR 96-well Armadillo plates (Thermo Fisher Scientific p/n AB2396)
Agilent shallow well reservoirs • used when NGS Workstation setup calls for **Agilent Shallow Well Reservoir**	Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 19 mm height (Agilent p/n 201254-100)
Agilent deep well reservoirs • used when NGS Workstation setup calls for **Agilent DW Reservoir**	Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 44 mm height (Agilent p/n 201244-100)
Agilent deep well plates • used when NGS Workstation setup calls for **Agilent DW Plate**	Agilent Storage/Reaction Microplates, 96 wells, 1 mL/round well (Agilent p/n 203426-100)
Agilent waste plates • used when NGS Workstation setup calls for **Waste Plate (Agilent 2 mL Square Well)**	Agilent Storage/Reaction Microplates, 96 wells, 2 mL/square well (Agilent p/n 201240-100)
Lids for plates and reservoirs	Hamilton PCR ComfortLid, p/n 814300

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)		
Post-Capture **Pooling**	SureSelect Custom Tier1 1–499 kb (96 Hybs)		
	SureSelect Custom Tier2 0.5–2.9 Mb (96 Hybs)		
	SureSelect Custom Tier3 3–5.9 Mb (96 Hybs)		
	SureSelect Custom Tier4 6–11.9 Mb (96 Hybs)		
	SureSelect Custom Tier5 12–24 Mb (96 Hybs)		
	Agilent Community Designs: Please visit the Community Designs (NGS) webpage at agilent.com for information on custom panels developed in collaboration with experts in various fields.	Design details and ordering information are available at the SureDesign website on the *Published Designs* tab. Contact the SureSelect support team (see **page 2**) or your local representative if you need assistance.	
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 XT HS PreCap Clinical Research Exome V4 (12 Hybs)	N/A	Agilent p/n 5280-0025
	SureSelect Pre-Capture Pooling Human All Exon V7 (12 Hybs)	S31285117	Agilent p/n 5191-5735
Post-Capture **Pooling**	SureSelect XT HS Human All Exon V8 (96 Hybs)	S33266340	Agilent p/n 5191-6875
	SureSelect XT HS Human All Exon V8+UTR (96 Hybs)	S33613271	Agilent p/n 5191-7403
	SureSelect XT HS Human All Exon V8+NCV (96 Hybs)	S33699751	Agilent p/n 5191-7409
	SureSelect XT HS Clinical Research Exome V4 (96 Hybs)	S34226467	Agilent p/n 5280-0022
	SSel XT HS and XT Low Input Human All Exon V7 (96 Hybs)	S31285117	Agilent p/n 5191-4029

NOTE

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

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

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

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

Table 6 Nucleic Acid Analysis Platform Options -- Select One

Description	Vendor and part number
Agilent 4200/4150 TapeStation Instrument	Agilent p/n G2991AA/G2992AA
Consumables:	
96-well sample plates	p/n 5042-8502
96-well plate foil seals	p/n 5067-5154
8-well tube strips	p/n 401428
8-well tube strip caps	p/n 401425
D1000 ScreenTape	p/n 5067-5582
D1000 Reagents	p/n 5067-5583
High Sensitivity D1000 ScreenTape	p/n 5067-5584
High Sensitivity D1000 Reagents	p/n 5067-5585
Agilent 2100 Bioanalyzer Instrument	p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	p/n G2953CA
Consumables:	
DNA 1000 Kit	p/n 5067-1504
High Sensitivity DNA Kit	p/n 5067-4626
Agilent 5200/5300/5400 Fragment Analyzer Instrument	Agilent p/n M5310AA/M5311AA/M5312AA
Consumables:	
NGS Fragment Kit (1-6000bp)	p/n DNF-473-0500
HS NGS Fragment Kit (1-6000bp)	p/n DNF-474-0500

Table 7 Recommended supplies for decontaminating the NGS Workstation surfaces

Description	Vendor
Dilute bleach (10%) wipes	Hype-Wipe Bleach Towelettes, or equivalent
Isopropanol (70%) wipes	VWR Pre-Moistened Clean Wipes, or equivalent

2 Overview of the Agilent Bravo NGS Workstation Option B+

This chapter contains an orientation to the Agilent Bravo NGS Workstation Option B+ and considerations for designing SureSelect experiments for automated processing using the NGS Workstation Option B+.

About the Agilent Bravo NGS Workstation Option B+

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

The Agilent Bravo NGS Workstation Option B+ is built on the Bravo automated liquid handling robot preconfigured for library prep and target enrichment when using next-generation sequencing (NGS) protocols. The Option B+ configuration integrates all of the accessories of the original NGS Workstation — including the Bravo automated liquid handling robot, the Labware MiniHub, and the BenchCel microplate handler — while also adding a thermal cycler on the Bravo deck. As part of the Option B+ system, the thermal cycler is programmed and controlled through the VWorks software, thus eliminating the need to transfer plates between the Bravo deck and a separate thermal cycler instrument.

Review the user guides listed in **Table 8** (available at www.agilent.com) to become familiar with the general features and operation of the NGS Workstation Option B+ components. Instructions for using the Bravo platform and other workstation components specifically for the SureSelect XT HS2 Target Enrichment workflow are detailed in this user guide.

Table 8 NGS Workstation components User Guide reference information

Device	User Guide part number
Bravo Platform	D0004797
VWorks Software	D0004378 (VWorks User Guide) D0004028 (VWorks Standard Quick Reference) D0004029 (VWorks Plus Quick Reference)
BenchCel Microplate Handler	G5580-90000
Labware MiniHub	G5584-90001
PlateLoc Thermal Microplate Sealer	G5585-90010
On-Deck Thermal Cycler (ODTC)	D0024535

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.

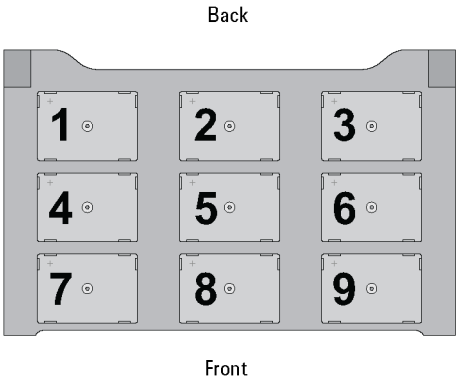


Figure 1 Bravo platform deck

On-Deck Thermal Cycler (ODTC)

The NGS Workstation Option B+ includes an ODTC at Bravo deck position 2. The ODTC has a 96-well thermal block that can maintain temperatures from 4°C to 99°C, and a heated lid that is capable of reaching 115°C.

The automated protocols designed for the NGS Workstation Option B+ use the ODTC for incubation and thermal cycling steps. The compatible SureSelect forms in the VWorks software include settings for parameters such as PCR cycle number and PCR plate type.

Instructions on setting up, maintaining, and troubleshooting the ODTC are provided in the Bravo ODTC User Guide (Agilent publication number D0024535).

Setting the Temperature of Bravo Deck Heat Blocks

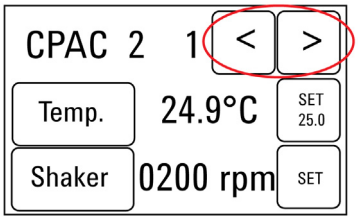
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

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

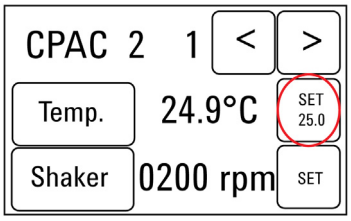
Table 9 Inheco Multi TEC Control touchscreen designations

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

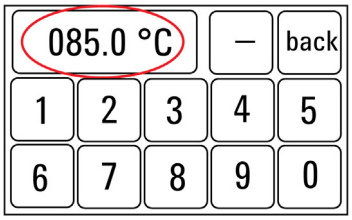
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



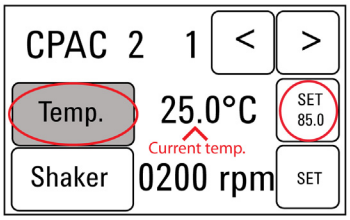
2 To set the temperature of the selected block, press the SET button.



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



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



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

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

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

1. Turn on the ThermoCube and wait for the LCD screen to display TEMP.
2. Press the UP or DOWN button to change SET TEMP 1 to the required set point.
3. Press the START button.

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

VWorks Automation Control Software

VWorks software, included with your Agilent Bravo NGS Workstation Option B+, allows you to control the robot and integrated devices using a PC. The NGS Workstation Option B+ is preloaded with VWorks software containing all of the necessary SureSelect system liquid handling protocols.

General instructions and introductory information on the VWorks software are provided below. For information specific to the SureSelect XT HS2 DNA form, see **"Using the SureSelect XT HS2 DNA Form"** on page 33.

NOTE

The instructions in this manual are compatible with VWorks software version 14.2.0.1534.

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

Accessing the SureSelect XT HS2 DNA form in the VWorks software

1. Start the SiLA XML RPC server. *This step is required for connecting the VWorks software to the NGS Workstation hardware.*
   a. From the Windows Start menu, launch SiLA XML RPC Server.
   b. Click **Start server**.
2. Launch the VWorks software by double-clicking the VWorks shortcut on the Windows desktop.

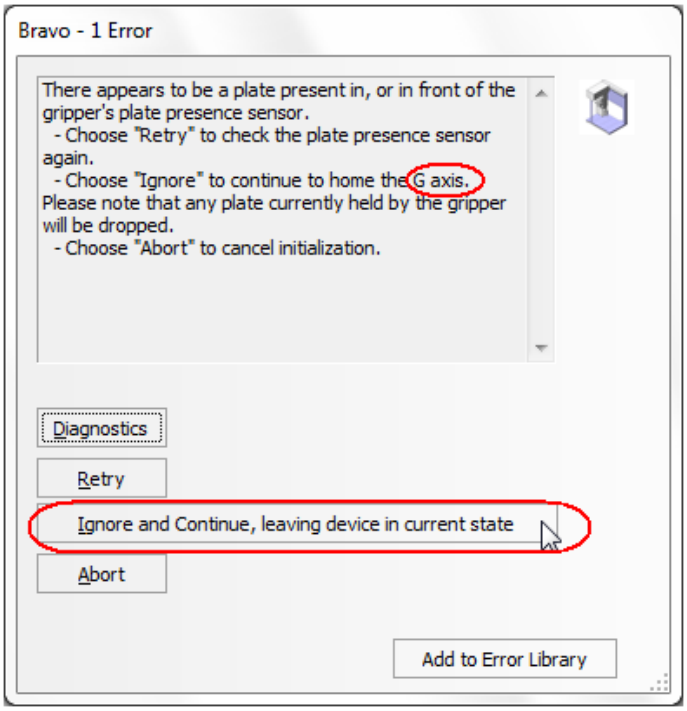
VWorks protocol and runset files

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

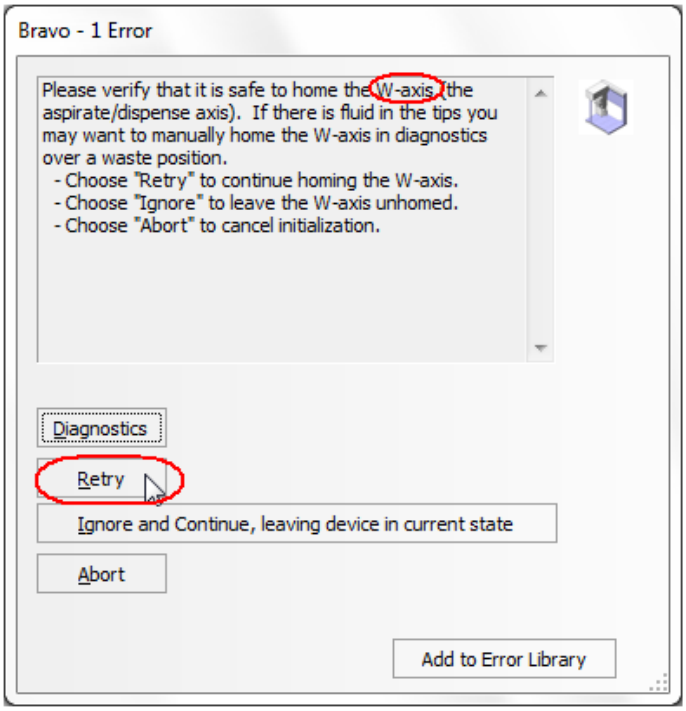
Error messages encountered at start of run

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

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



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



Verifying the Simulation setting

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

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

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

NOTE

If you cannot see the toolbar above the SureSelect_XT_HS2_ILM 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.

Experimental Setup Considerations for Automated Runs

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

Table 10 Columns to Samples Equivalency

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

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

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

Refer to **Figure 2** on page 29 for an overview of the workflow.

- The NGS Workstation processes samples column-wise beginning at column 1. The gDNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- At the hybridization step, you can add a different Probe to each row of the plate. Plan your experiment such that each prepared DNA library corresponds to the appropriate Probe row in the sample plate.
- For post-capture amplification, different Probes can require different amplification cycle numbers, based on the Probe design sizes. All Probes being processed in the same run need to use the same post-capture PCR cycle number. See **Table 66** on page 93 to determine which Probes may be amplified on the same plate.

Decontamination Procedure

After running a runset or protocol that includes PCR amplification, decontaminate the surfaces of the NGS Workstation using the procedure described below. See **Table 7** on page 16 for the recommended supplies.

The runsets for library preparation and for hybridization and capture include PCR amplification steps.

1. Turn off power to the Bravo platform and ODTC.
2. Use a pre-moistened dilute bleach wipe (10% bleach) to wipe down the exposed surfaces of the Bravo deck.
3. Wipe down these same areas again using a wipe pre-moistened with 70% isopropanol.
4. Allow the surfaces to dry before using the NGS Workstation.

Refer to the Bravo ODTC User Guide (Agilent publication D0024535) and the Bravo Platform User Guide (Agilent publication D0004797) for guidance on device cleaning.

3 Overview of SureSelect XT HS2 DNA Automation

This chapter contains an overview of the SureSelect XT HS2 DNA target enrichment workflow and the SureSelect XT HS2 DNA VWorks form for the NGS Workstation Option B+.

Overview of the SureSelect XT HS2 DNA Workflow

Figure 2 summarizes the SureSelect XT HS2 library preparation and target enrichment workflow for genomic DNA (gDNA) samples to be sequenced using the Illumina paired-read sequencing platform.

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 129). 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 12** through **Table 14** for summaries of the VWorks runsets and protocols used during the workflow. The chapters on **Preparation of AMPure XP Bead Plates**, **DNA Fragmentation and Library Preparation**, **Target Enrichment**, and **Sequencing Preparation and Processing** contain complete instructions for executing the workflow, including how to set up and run the VWorks runsets and protocols.

The SureSelect XT HS2 DNA workflow is compatible with both high-quality DNA 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.

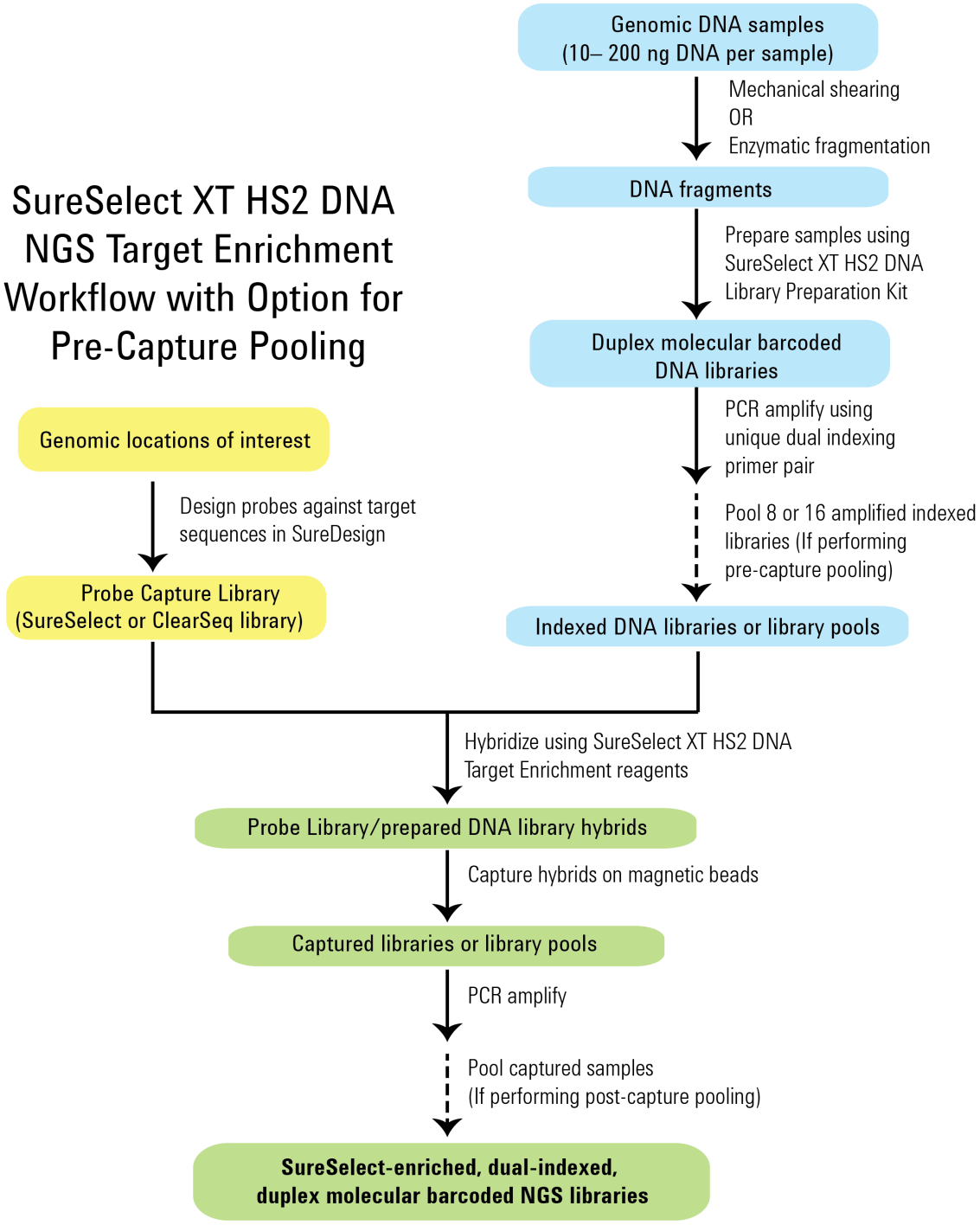


Figure 2 Overall sequencing sample preparation workflow

Workflow Modulations

The SureSelect XT HS2 DNA target enrichment workflow can be modulated for different applications as described below and summarized in **Table 11** on page 30.

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

DNA Fragmentation Method DNA can be fragmented enzymatically or mechanically. The choice determines which library preparation runset is required. See **Figure 6** 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 11 Summary of workflow modulations supported by the automation protocols

Property	Options	Usage Notes
DNA Sample Integrity	Intact DNA	Use standard protocol with 10–200 ng input DNA
	FFPE DNA	Qualify DNA before use in assay; see **"Protocol modifications for FFPE Samples"** on page 114 for summary of protocol modifications.
DNA Fragmentation Method	Enzymatic Fragmentation	Use the EnzymaticFrag_LibPrep_XT_HS2 runset to perform enzymatic fragmentation of DNA samples. The runset executes the steps for enzymatic fragmentation as well as library preparation. Requires purchase of SureSelect Enzymatic Fragmentation Kit, 96 Reactions Automation (Agilent p/n 5191-6764)
	Mechanical Fragmentation (Covaris)	Perform mechanical shearing on Covaris Sample Preparation System using manual liquid handling (no automated protocol). Then, continue by running the MechanicalFrag_LibrPrep_XT_HS2 runset to execute the steps for library preparation. Requires purchase of Covaris Sample Preparation System and consumables (see **"Additional Required Materials based on DNA Sample Type/Fragmentation Method"** on page 15).
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 13.
	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 13.

Automation Runsets and Protocols used in the Workflow

The workflow uses runsets and protocols that are executed from the SureSelect XT HS2 DNA VWorks form and from the supplemental VWorks forms (utility form and pooling and normalization form). The runsets and protocols that are available on each form are described in **Table 12** through **Table 14**.

Note that the supplemental forms are accessible from within the SureSelect XT HS2 DNA VWorks form. See **"Accessing the Supplemental Forms"** on page 36.

Table 12 SureSelect XT HS2 DNA VWorks form - Options in *Select runset to execute*

Runset name	Description
DNA Fragmentation & Library Preparation	
EnzymaticFrag_LibPrep_XT_HS2	For use with workflows that use enzymatic fragmentation of DNA. This runset shears DNA samples using enzymatic fragmentation with restriction enzymes, then prepares purified indexed DNA libraries for either multi- or single-plexed hybridization.
MechanicalFrag_LibPrep_XT_HS2	For use with workflows that use mechanical fragmentation of DNA. Starting with DNA that has already been fragmented on the Covaris Sample Preparation System, this runset prepares purified indexed DNA libraries for either multi-plexed hybridization or single-plexed hybridization.
Target Enrichment	
SSELHyb&Capture_XT_HS2	This runset hybridizes prepped libraries or library pools to Probe (multi-plexed or single-plexed), then prepares amplified, purified libraries or library pools.

Table 13 VWorks utility form - Options in *Select protocol to execute*

Protocol name	Description
AMPureXP Aliquot for XT HS2	
AMPureXP_Aliquot (Case: Library Prep)	Prepares the plate of aliquoted AMPure XP beads needed for library prep purification. The plate is required for setup of the DNA fragmentation & library preparation runsets.
AMPureXP_Aliquot (Case: Pre-Capture PCR)	Prepares the plate of aliquoted AMPure XP beads needed for pre-capture PCR purification. The plate is required for setup of the DNA fragmentation & library preparation runsets.
AMPureXP_Aliquot (Case: Concentration of Pool)	For use with pre-capture pooling workflows. Prepares the plate of aliquoted AMPure XP Beads needed for concentrating the DNA during pre-capture pooling. The plate is required for setup of the AMPureXP_XT_HS2 (Case Concentration of Pool) protocol.
AMPureXP_Aliquot (Case: Post-Capture PCR)	Prepares the plate of aliquoted AMPure XP Beads needed for post-capture PCR purification. The plate is required for setup of the SSELHyb&Capture_XT_HS2 runset.
Single-Plex Pre-Hybridization (for use with post-capture pooling workflows)	
Aliquot_Libraries	Prepares a plate containing 500–1000 ng aliquots of prepped libraries.
Multi-Plex Pre-Hybridization (for use with pre-capture pooling workflows)	
Aliquot_Water	Aliquots the required volume of water for each library pool in order to normalize pool volumes to 100 µL.
AMPureXP_XT_HS2_ILM (Case: Concentration of Pool)	Concentrates pooled samples to 24 µL.

Table 13 VWorks utility form - Options in *Select protocol to execute*

Protocol name	Description
TapeStation Setup	
TS_D1000	Prepares the analysis plate for analyzing indexed DNA libraries using Agilent TapeStation platform.
TS_HighSensitivity_D1000	Prepares the analysis plate for analyzing final libraries or library pools using Agilent TapeStation platform.
Post-Capture Pooling (for use with post-capture pooling workflows)	
Aliquot_Captures	Pools indexed DNA libraries prior to sequencing. (Performed after the Aliquot_Water protocol.)

Table 14 Pooling and Normalization VWorks form

Protocol name	Description
XT_HS2_Pooling protocol	For use in pre-capture pooling workflows. Pools indexed DNA libraries in pools of 8 or 16.

Using the SureSelect XT HS2 DNA Form

Use the SureSelect XT HS2 DNA VWorks form for the NGS Workstation Option B+ to set up and start each SureSelect automation runset.

Overview of the SureSelect XT HS2 DNA form

Refer to **Figure 3** for an overview of the SureSelect XT HS2 DNA form.

- The left side of the form has a variety of tools and controls (drop-down lists, fields, buttons, etc.) for selecting the runset, selecting the number of columns in the run, defining the parameters of your workflow, starting or pausing a run, and accessing supplemental forms and other resources.
- The right side of the form has graphic representations of the Bravo deck (positions 1 through 9), the BenchCel microplate handler (stackers 1 through 4), and the Labware MiniHub (Cassettes 1 through 4 and shelves 5 through 1).

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

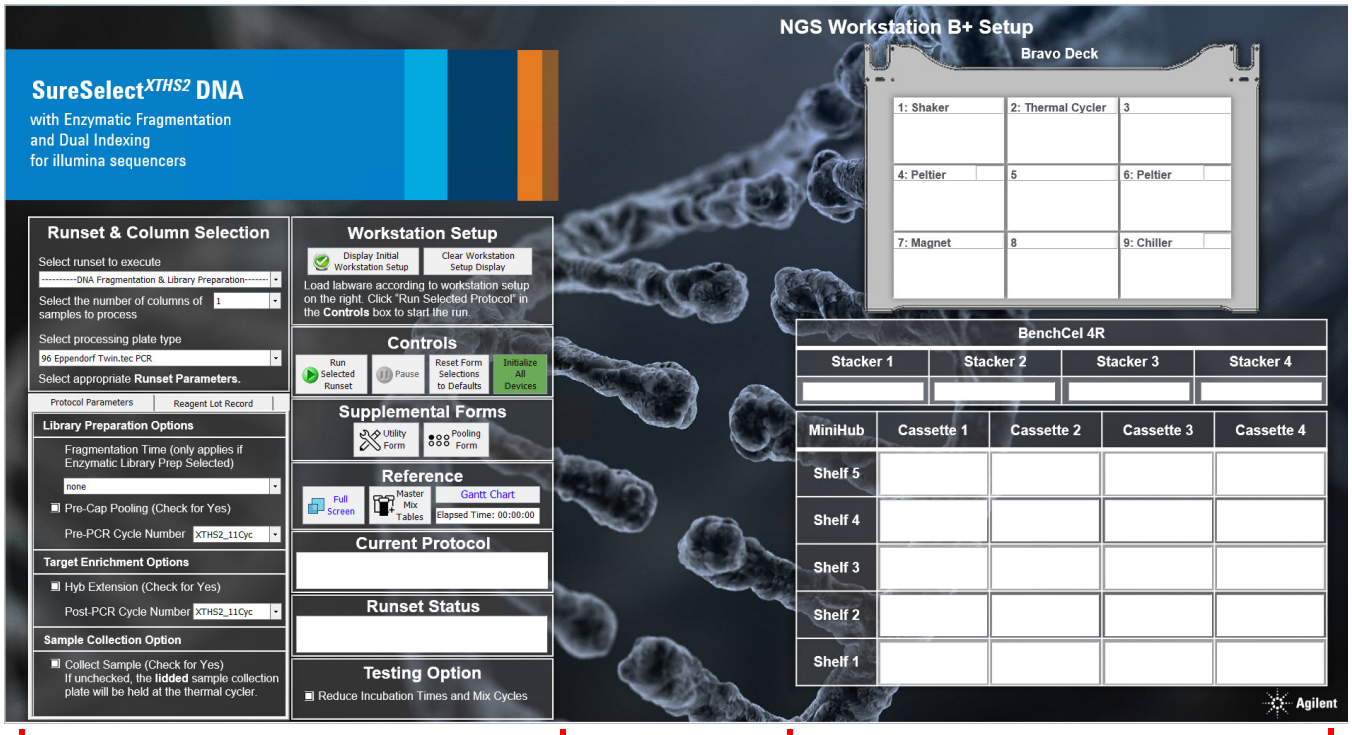


Figure 3 Overview of the SureSelect XT HS2 DNA form for the NGS Workstation Option B+

Opening the SureSelect XT HS2 DNA form

1 Start the SiLA XML RPC server.

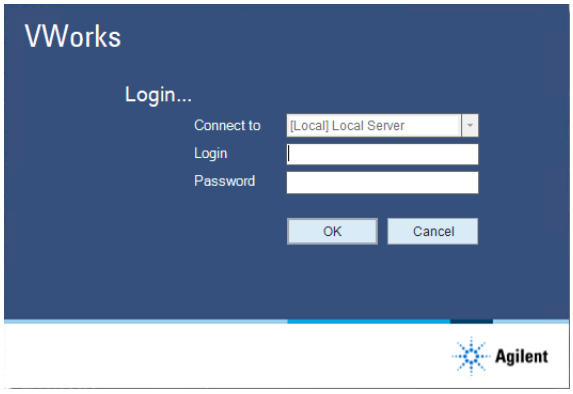
   a From the Windows Start menu, launch SiLA XML RPC Server.

   b Click **Start server**.

   c Leave the SiLA XML RPC Server window open while using the SureSelect XT HS2 DNA form.

2 Launch the VWorks software by double-clicking the VWorks shortcut on the Windows desktop.

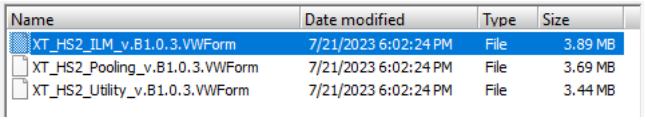
3 In the Login dialog box, enter your login user name and password, and click **OK**.



4 In VWorks, click **File > Open > Form**.

   The Open File dialog box opens.

5 Select **XT_HS2_ILM_v.B1.0.3.VWForm** (as shown below) and click **Open**.

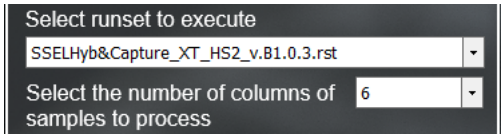


   The SureSelect XT HS2 DNA form opens in VWorks.

Steps for using the SureSelect XT HS2 DNA form

The instructions below provide the basic steps for setting up and executing a runset on the SureSelect XT HS2 DNA form. The chapters on **"DNA Fragmentation and Library Preparation"** and **"Target Enrichment"** contain detailed setup instructions for each runset.

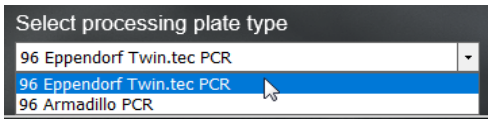
1 Under **Runset & Column Selection**, use the drop-down lists to select the runset to be executed and number of columns of samples to process in the run.



   Descriptions of the available runsets are provided in **Table 12** on page 31.

2 In the **Select processing plate type** drop-down list, verify that the correct plate type is selected.

The processing plate is either a 96-well Eppendorf twin.tec plate (Eppendorf p/n 951020401) or a 96-well Armadillo plate (Thermo Fisher Scientific p/n AB2396). **Do not substitute another plate type.** Use the same type of processing plate (Eppendorf twin.tec or Armadillo) for all protocols and runsets in the same run.



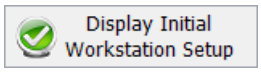
3 On the Protocol Parameters tab of the form, make the appropriate selections for **Library Preparation Options**, **Target Enrichment Options**, and **Sample Collection Option**.

These options are described in detail as part of the instructions for setting up individual runsets in the chapters on **"DNA Fragmentation and Library Preparation"** and **"Target Enrichment"**.

4 (Optional) On the Reagent Lot Record tab, enter the lot numbers of the reagents into the provided fields.

The entries in these fields are stored in the log file for the run.

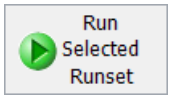
5 Once all run parameters have been specified on the form, click **Display Initial Workstation Setup**.



The graphic representations of the workstation on the right side of the form are populated with the required placement of reaction components and labware for the specified run parameters.

6 Load the NGS Workstation according to the form.

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



A dialog box opens asking you to confirm that the workstation is setup correctly before proceeding.

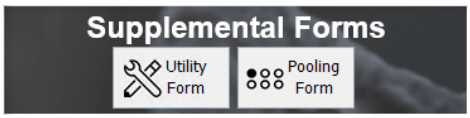
8 Click **OK** in the dialog box to initiate the runset.

The NGS Workstation executes the set of protocols in the runset until all protocols are completed.

9 After the run, collect the sample plate from the NGS Workstation as instructed by the software.

Accessing the Supplemental Forms

The SureSelect XT HS2 DNA VWorks form provides buttons, shown below, for accessing two supplemental forms: the Utility form and the Pooling and Normalization form.



Overview of the Utility form

To open the Utility form from the SureSelect XT HS2 DNA VWorks form, click **Utility Form** in the Supplemental Forms section. Once you are viewing the Utility form, you can return to the SureSelect XT HS2 DNA VWorks form by clicking **XT HS2 DNA Form** in the Supplemental Forms section.

The Utility form provides automated protocols to perform a variety of basic liquid-handling tasks used throughout the workflow. The tasks executed by these protocols can be, if desired, performed manually (i.e., without the use of an automated protocol) without significantly increasing the hands-on time of the workflow. Descriptions of the available protocols are provided in **Table 13** on page 31.

Refer to **Figure 4** for an overview of the Utility form.

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

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

Detailed setup instructions for the protocols on the Utility form are included as part of the instructions in the chapters on **"DNA Fragmentation and Library Preparation"** and **"Target Enrichment"**.

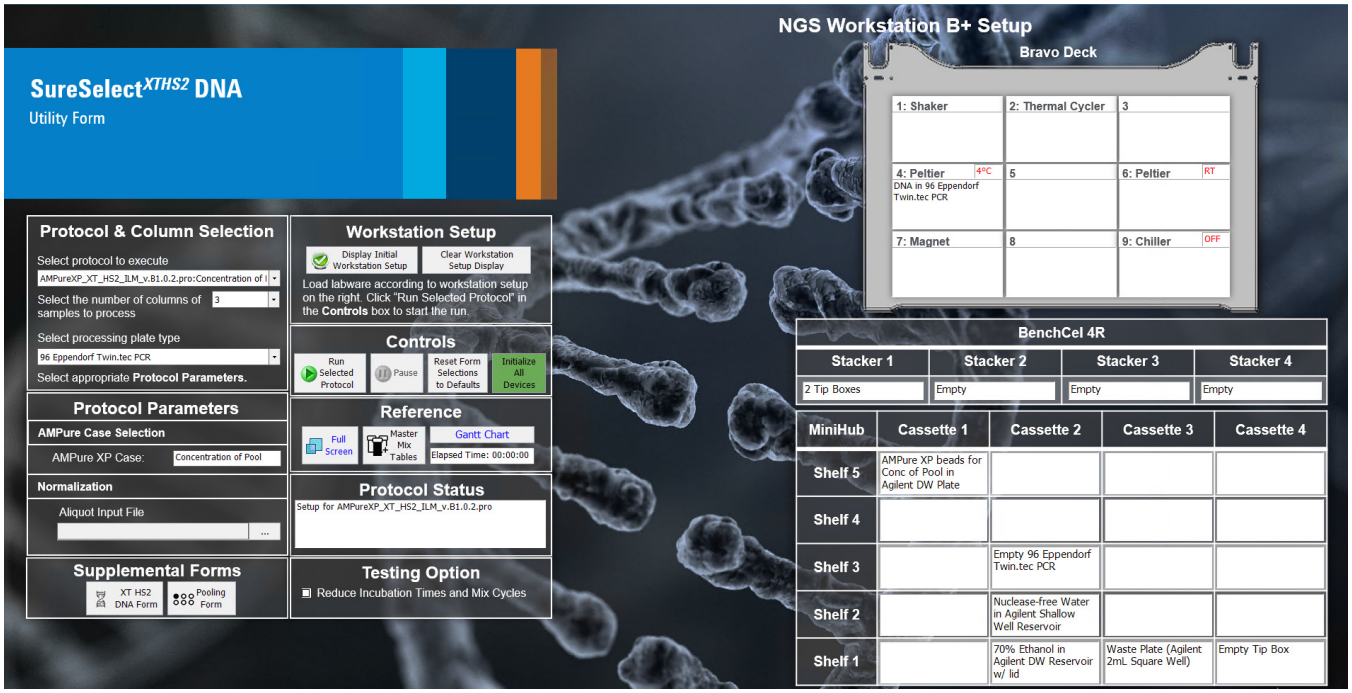


Figure 4 Overview of the Utility form for the NGS Workstation Option B+

Overview of the Pooling and Normalization form

To open the Pooling and Normalization form from the SureSelect XT HS2 DNA VWorks form, click **Pooling Form** in the Supplemental Forms section. Once you are viewing the Pooling and Normalization form, you can return to the SureSelect XT HS2 DNA VWorks form by clicking **XT HS2 DNA Form** in the Supplemental Forms section.

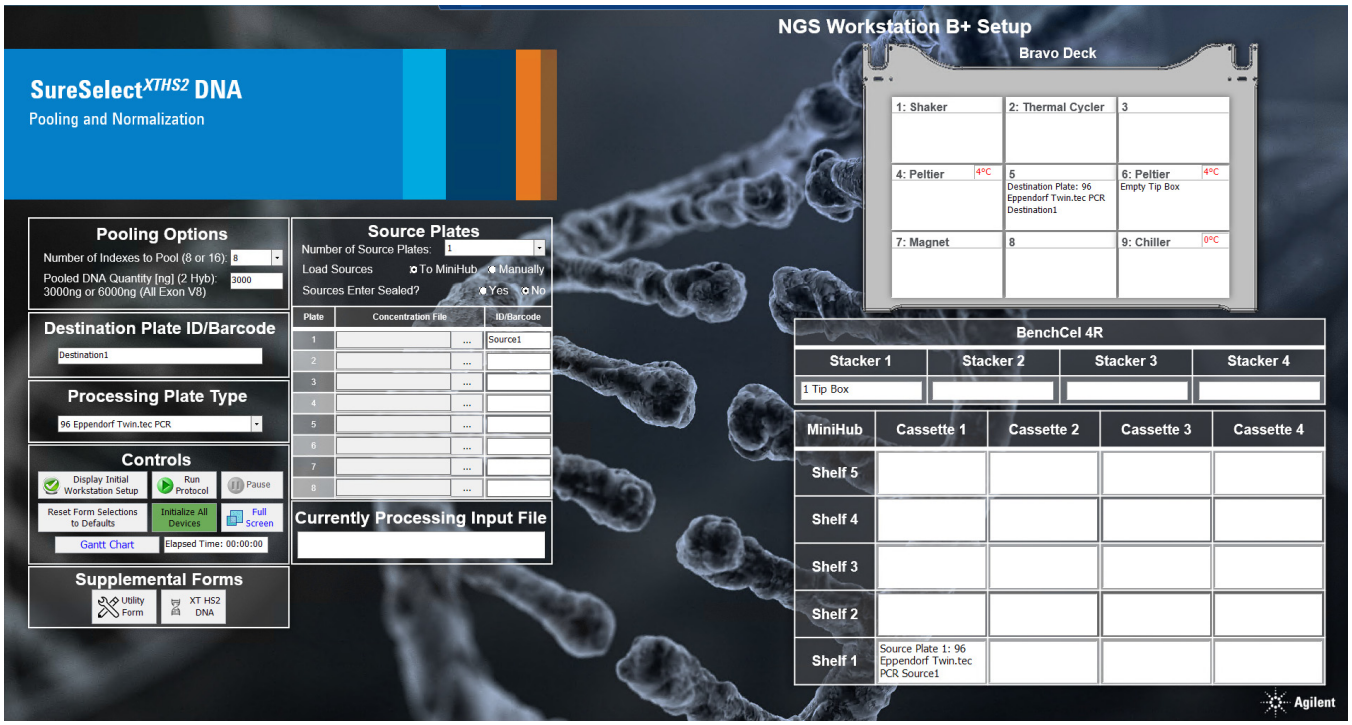
The Pooling and Normalization form provides an automated protocol for pooling 8 or 16 samples for the pre-capture pooling workflow. The pooling protocol is the only protocol offered through the Pooling and Normalization form.

Refer to **Figure 5** for an overview of the Pooling and Normalization form.

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

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

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



Tools and controls for making selections, starting/pausing runs, and accessing resources

Graphic representations of workstation setup

Figure 5 Overview of the Utility form for the NGS Workstation Option B+

4 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 VWorks Utility 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 library preparation bead plate

The library preparation runsets (EnzymaticFrag_LibPrep_XT_HS2 and MechanicalFrag_LibPrep_XT_HS2) require a bead plate for library prep purification that contains 80 µL of beads in each well. Use the AMPureXP_Aliquot (Case: Library Prep) protocol to prepare the library preparation bead plate.

Load the NGS Workstation for protocol AMPureXP_Aliquot (Case: Library Prep)

1. 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 Library Prep runset (each column accommodates 8 DNA samples).
2. Load the Bravo deck according to **Table 15**.

Table 15 Initial Bravo deck configuration for AMPureXP_Aliquot (Case: Library Prep) protocol

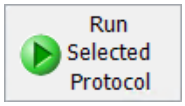
Location	Content
5	Empty tip box
6	Empty Agilent deep well plate
8	New tip box
9	Reservoir of AMPure XP bead suspension prepared in **step 1**

Run VWorks protocol AMPureXP_Aliquot (Case: Library Prep)

1. In the VWorks software, open the Utility form. See **"Accessing the Supplemental Forms"** on page 36.
2. Under **Select protocol to execute**, select the **AMPureXP_Aliquot (Case: Library Prep)** protocol.
3. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
4. Click **Display Initial Workstation Setup**.

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

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



Running the AMPureXP_Aliquot (Case: Library Prep) protocol takes approximately 5 minutes. The protocol directs the NGS Workstation to aliquot 80 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent deep well plate.

7 When the protocol is complete, remove the Agilent deep well plate containing the AMPure XP bead suspension from position 6 of the Bravo deck.

8 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 library preparation runset (refer to **Table 30** 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 library preparation runsets (EnzymaticFrag_LibPrep_XT_HS2 and MechanicalFrag_LibPrep_XT_HS2) require a bead plate for purification of pre-capture PCR products that contains 50 µL of beads in each well. Use the AMPureXP_Aliquot (Case: Pre-Capture PCR) protocol to prepare the pre-capture PCR bead plate.

Load the NGS Workstation for protocol AMPureXP_Aliquot (Case: Pre-Capture PCR)

1. 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 Library Prep runset (each column accommodates 8 amplified DNA samples).
2. Load the Bravo deck according to **Table 16**.

Table 16 Initial Bravo deck configuration for AMPureXP_Aliquot (Case: Pre-Capture PCR) protocol

Location	Content
5	Empty tip box
6	Empty Agilent deep well plate
8	New tip box
9	Reservoir of AMPure XP bead suspension prepared in **step 1**

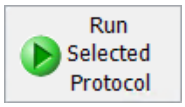
Run VWorks protocol AMPureXP_Aliquot (Case: Pre-Capture PCR)

1. In the VWorks software, open the Utility form. See **"Accessing the Supplemental Forms"** on page 36.
2. Under **Select protocol to execute**, select the **AMPureXP_Aliquot (Case: Pre-Capture PCR)** protocol.
3. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

4 Click **Display Initial Workstation Setup**.

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

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



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

7 When the protocol is complete, remove the Agilent deep well plate containing the AMPure XP bead suspension from position 6 of the Bravo deck.

8 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 library preparation runset (refer to **Table 30** 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 3. Prepare the bead plate to be used for concentration of pooled DNA (pre-capture pooling workflow only)

The AMPureXP_XT_HS2_ILM (Case: Concentration of Pool) protocol is part of the pre-capture pooling workflow. It requires a bead plate for concentrating the DNA library pools that contains 180 µL of beads in each well. Use the AMPureXP_Aliquot (Case: Concentration of Pool) protocol to prepare the concentration of pool bead plate.

The bead plate for the AMPureXP_XT_HS2_ILM (Case: Concentration of Pool) protocol is only needed if you are running the pre-capture pooling workflow option. If you are using the post-capture pooling workflow, proceed to **"Step 4. Prepare the bead plate to be used for Post-Capture Purification"** on page 46.

Load the NGS Workstation for protocol AMPureXP_Aliquot (Case: Concentration of Pool)

1. 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 AMPureXP_XT_HS2_ILM (Case: Concentration of Pool) protocol (each column accommodates 8 DNA library pools).
2. Load the Bravo deck according to **Table 17**.

Table 17 Initial Bravo deck configuration for AMPureXP_Aliquot (Case: Concentration of Pool) protocol

Location	Content
5	Empty tip box
6	Empty Agilent deep well plate
8	New tip box
9	Reservoir of AMPure XP bead suspension prepared in **step 1**

Run VWorks protocol AMPureXP_Aliquot (Case: Concentration of Pool)

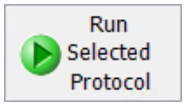
1. In the VWorks software, open the Utility form. See **"Accessing the Supplemental Forms"** on page 36.
2. Under **Select protocol to execute**, select the **AMPureXP_Aliquot (Case: Concentration of Pool)** protocol.

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

4 Click **Display Initial Workstation Setup**.

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

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



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

7 When the protocol is complete, remove the Agilent deep well plate containing the AMPure XP bead suspension from position 6 of the Bravo deck.

8 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 **AMPureXP_XT_HS2_ILM (Case: Concentration of Pool)** protocol (refer to **Table 49** on page 83). 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 SSELHyb&Capture_XT_HS2 runset for target enrichment requires a bead plate for purification of post-capture PCR products that contains 50 µL of beads in each well. Use the AMPureXP_Aliquot (Case: Post-Capture PCR) protocol to prepare the post-capture PCR bead plate.

Load the NGS Workstation for protocol AMPureXP_Aliquot (Case: Post-Capture PCR)

1 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 SSELHyb&Capture_XT_HS2 runset (each column accommodates 8 indexed libraries).

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

Table 18 Initial Bravo deck configuration for AMPureXP_Aliquot (Case: Post-Capture PCR) protocol

Location	Content
5	Empty tip box
6	Empty Agilent deep well plate
8	New tip box
9	Reservoir of AMPure XP bead suspension prepared in **step 1**

Run VWorks protocol AMPureXP_Aliquot (Case: Post-Capture PCR)

1 In the VWorks software, open the Utility form. See **"Accessing the Supplemental Forms"** on page 36.

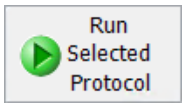
2 Under **Select protocol to execute**, select the **AMPureXP_Aliquot (Case: Post-Capture PCR)** protocol.

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

4 Click **Display Initial Workstation Setup**.

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

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



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

7 When the protocol is complete, remove the Agilent deep well plate containing the AMPure XP bead suspension from position 6 of the Bravo deck.

8 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 SSELHyb&Capture_XT_HS2 runset (refer to **Table 62** on page 92). 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.

5 DNA Fragmentation and Library Preparation

This chapter provides instructions for DNA fragmentation and DNA library preparation for the Illumina paired-read sequencing platform. For each sample to be sequenced, an individual indexed and molecular-barcoded library is prepared.

For an overview of the SureSelect XT HS2 target enrichment workflow, see **Figure 2** on page 29.

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 8** on **page 113**.

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 **"Step 2. Fragment DNA by Mechanical Shearing"** on page 52 or **"Step 3. Perform Library Preparation"** 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 19**.

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 19 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 20** to determine the recommended amount of input DNA for the sample.

Table 20 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 DNA by Mechanical Shearing

CAUTION The SureSelect XT HS2 DNA workflow for the NGS Workstation Option B+ supports two different methods for preparing fragmented DNA (mechanical shearing and enzymatic fragmentation). Perform this step only if you are using mechanical shearing to fragment the gDNA samples. If you are using enzymatic fragmentation, proceed to **"Step 3. Perform Library Preparation"** on page 54.

In this step, 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 21** for guidelines. Complete shearing instructions are provided on **page 53**.

Table 21 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 19** or **Table 20** 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.

a 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.

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

c Load the 96 microTUBE Plate onto the loading tray and shear the DNA with the settings in **Table 22**.

Table 22 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

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

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

f 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 e**.

g Cover the plate of sheared DNA samples with a lid and keep on ice.

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 f**.

Step 3. Perform Library Preparation

The SureSelect XT HS2 DNA VWorks form for the NGS Workstation Option B+ provides two different runsets for library preparation. The choice of runset depends on which method of DNA fragmentation you are using in your workflow. Both runsets result in a sample plate containing molecular-barcoded, indexed DNA libraries that have been PCR-amplified using unique dual-indexing primer pairs and then purified using AMPure XP beads.

Enzymatic fragmentation When using enzymatic fragmentation, a single runset (called **EnzymaticFrag_LibPrep_XT_HS2**) executes the automated protocols for enzymatic fragmentation of the DNA samples followed by the automated protocols for library preparation.

Mechanical shearing When using mechanical shearing for fragmentation, you first fragment the DNA samples on the Covaris E220 instrument (as instructed in the previous section, **"Step 2. Fragment DNA by Mechanical Shearing"** on page 52). Then, the **MechanicalFrag_LibPrep_XT_HS2** runset executes the automated protocols for library preparation.

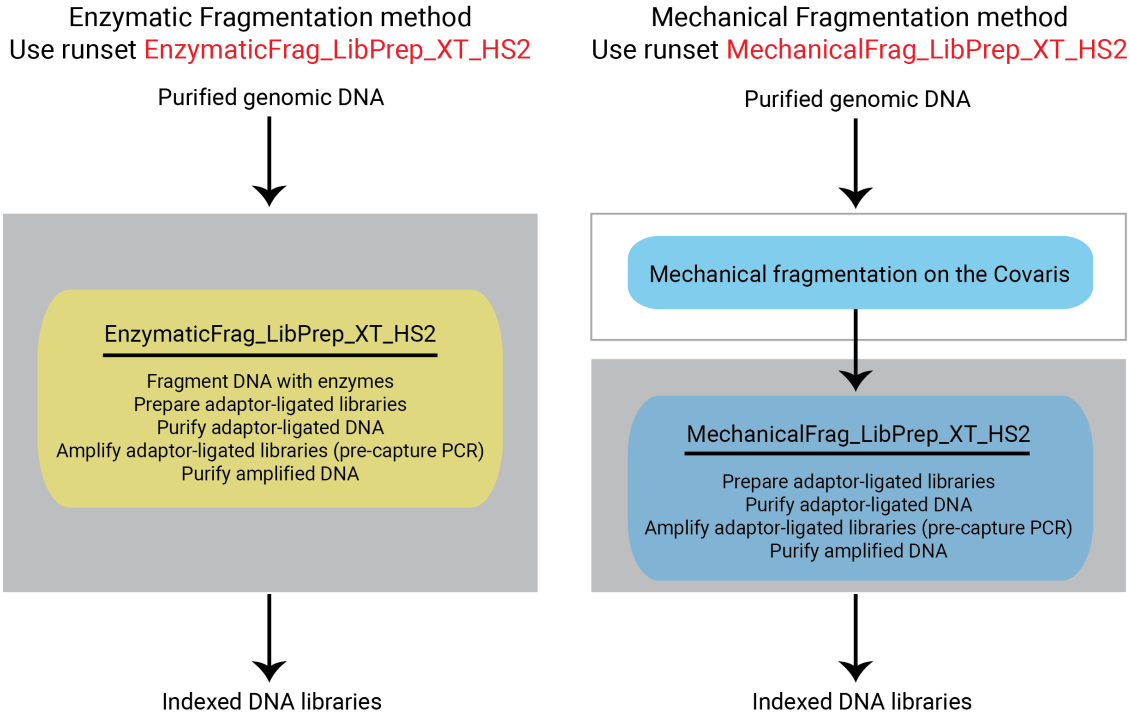


Figure 6 Library preparation runset options based on DNA fragmentation method.

NOTE The instructions for library preparation that are provided in this step differ at certain points depending on which fragmentation method you are using. These differences are noted in the text. As you follow the instructions, make sure you are following the instructions that apply to your chosen DNA fragmentation method.

This step uses the components listed in **Table 23**. Thaw and mix each component as directed in **Table 23** before use.

Table 23 Reagents thawed before use in runset

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
5X SureSelect Fragmentation Buffer (blue cap) *For enzymatic fragmentation workflow only*	SureSelect Enzymatic Fragmentation Kit, –20°C	Thaw on ice then keep on ice	Vortexing	**page 56**
SureSelect Fragmentation Enzyme (green cap) *For enzymatic fragmentation workflow only*	SureSelect Enzymatic Fragmentation Kit, –20°C	Place on ice just before use	Inversion	**page 56**
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**
SureSelect XT HS2 Index Primer Pairs	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR),* –20°C	—	Vortexing	**page 57**
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**
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**
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**
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Place on ice just before use	Pipette up and down 15–20 times	**page 59**
5× Herculase II Reaction Buffer (clear cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	**page 59**

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

Prepare the NGS Workstation and reagent reservoirs

1. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
2. 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 19.

   Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.

3 For enzymatic fragmentation workflows only: 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.

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

   Prepare an Agilent deep well reservoir containing 100 mL of freshly-prepared 70% ethanol. Add a lid to prevent evaporation.

Prepare the reagents needed for fragmentation

NOTE The steps in **"Prepare the reagents needed for fragmentation"** are only required for the enzymatic fragmentation workflow. If you used mechanical fragmentation to fragment the DNA samples, bypass this section and proceed directly to **"Prepare the SureSelect XT HS2 Index Primer Pairs"** on page 57.

Prepare the sample plate for fragmentation (for enzymatic fragmentation workflow only)

1 In the wells of a processing plate (Eppendorf twin.tec or Armadillo plate), dilute 10–200 ng of each gDNA sample with nuclease-free water to a final volume of 15 µL. Add a lid to the plate and keep on ice.

   See **Table 19** or **Table 20** for FFPE DNA input guidelines based on the measured DNA quality in each sample.

Prepare the Fragmentation master mix (for enzymatic fragmentation workflow only)

2 Prepare the appropriate volume of Fragmentation master mix by combining the reagents in **Table 24**.

   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 24 Preparation of Fragmentation master mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2 µL	42.5 µL	59.5 µL	76.5 µL	97.8 µL	136.0 µL	253.8 µL
5X SureSelect Fragmentation Buffer (blue cap)	2 µL	42.5 µL	59.5 µL	76.5 µL	97.8 µL	136.0 µL	253.8 µL
SureSelect Fragmentation Enzyme (green cap)	1 µL	21.3 µL	29.8 µL	38.3 µL	48.9µL	68.0 µL	126.9 µL
Total Volume	**5 µL**	**106.3 µL**	**148.8 µL**	**191.3µL**	**244.4 µL**	**340.0 µL**	**634.4 µL**

Prepare the SureSelect XT HS2 Index Primer Pairs

1 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 a processing plate (Eppendorf twin.tec or Armadillo plate). Add a lid to the plate and keep on ice.

Make sure to maintain the same well location for each primer pair when transferring to the PCR plate.

Prepare the library preparation master mixes

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

1 Prepare the appropriate volume of End Repair/dA-Tailing master mix, using volumes listed in Table 25 and using the liquid handling steps specified below.

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 25 Preparation of End Repair/dA-Tailing master mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
End Repair-A Tailing Buffer (yellow cap or bottle)	16 µL	204 µL	340 µL	476 µL	612 µL	884 µL	1836 µL
End Repair-A Tailing Enzyme Mix (orange cap)	4 µL	51 µL	85 µL	119 µL	153 µL	221 µL	459 µL
Total Volume	**20 µL**	**255 µL**	**425 µL**	**595 µL**	**765 µL**	**1105 µL**	**2295 µL**

Prepare the ligation master mix

2 Prepare the appropriate volume of Ligation master mix, using volumes listed in **Table 26** and using the liquid handling steps specified below.

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 26 Preparation of Ligation master mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Ligation Buffer (purple cap or bottle)	23 µL	293.3 µL	488.8 µL	684.3 µL	879.8 µL	1270.8 µL	2737 µL
T4 DNA Ligase (blue cap)	2 µL	25.5 µL	42.5 µL	59.5 µL	76.5 µL	110.5 µL	238 µL
Total Volume	**25 µL**	**318.8 µL**	**531.3 µL**	**743.8 µL**	**956.3 µL**	**1381.3 µL**	**2975 µL**

Prepare the Adaptor Oligo Mix

3 Prepare the appropriate volume of Adaptor Oligo Mix dilution, according to **Table 27**. Mix well using a vortex mixer and keep on ice.

Table 27 Preparation of Adaptor Oligo Mix dilution

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2.5 µL	42.5 µL	63.8 µL	85.0 µL	106.3 µL	143.5 µL	276.3 µL
SureSelect XT HS2 Adaptor Oligo Mix (white cap)	5 µL	85.0 µL	127.5 µL	170.0 µL	212.5 µL	287.0 µL	552.5 µL
Total Volume	**7.5 µL**	**127.5 µL**	**191.3 µL**	**255.0 µL**	**318.8 µL**	**430.5 µL**	**828.8 µL**

Prepare the pre-capture PCR master mix

4 Prepare the appropriate volume of pre-capture PCR master mix, according to **Table 28**. Vortex at medium speed for 15–20 seconds and keep on ice.

Table 28 Preparation of Pre-Capture PCR Master Mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
5× Herculase II Buffer with dNTPs (clear cap)	10 µL	170 µL	255 µL	340µL	425 µL	574 µL	1066 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17 µL	25.5 µL	34 µL	42.5 µL	57.4 µL	106.6 µL
Total Volume	**11 µL**	**187 µL**	**280.5 µL**	**374µL**	**467.5 µL**	**631.4 µL**	**1172.6 µL**

Prepare the master mix source plate

1 Using an Agilent deep well plate, prepare the master mix source plate for the run as indicated in **Table 29**. Add the indicated volume of the master mix to all wells of the indicated column of the plate. Keep the master mixes on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 7**.

Table 29 Preparation of the master mix source plate for the library prep runset

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Fragmentation master mix (if using)*	Column 1 (A1-H1)	12.5 µL	17.5 µL	22.5 µL	28.8 µL	40.0 µL	75.0 µL
End Repair-dA Tailing master mix	Column 2 (A2-H2)	31.0 µL	52.0 µL	73.0 µL	94.0 µL	136.0 µL	280.0 µL
Ligation master mix	Column 3 (A3-H3)	36.0 µL	62.0 µL	88.0 µL	114.0 µL	166.0 µL	360.0 µL
Adaptor Oligo Mix dilution	Column 4 (A4-H4)	15.0 µL	22.5 µL	30.0 µL	37.5 µL	52.5 µL	101.3 µL
Pre-Capture PCR Master Mix	Column 5 (A5-H5)	22 µL	33 µL	44 µL	55 µL	77 µL	143 µL

* The fragmentation master mix is only needed for the enzymatic fragmentation workflow. If you are using the mechanical fragmentation workflow, leave column 1 of the master mix source place empty.

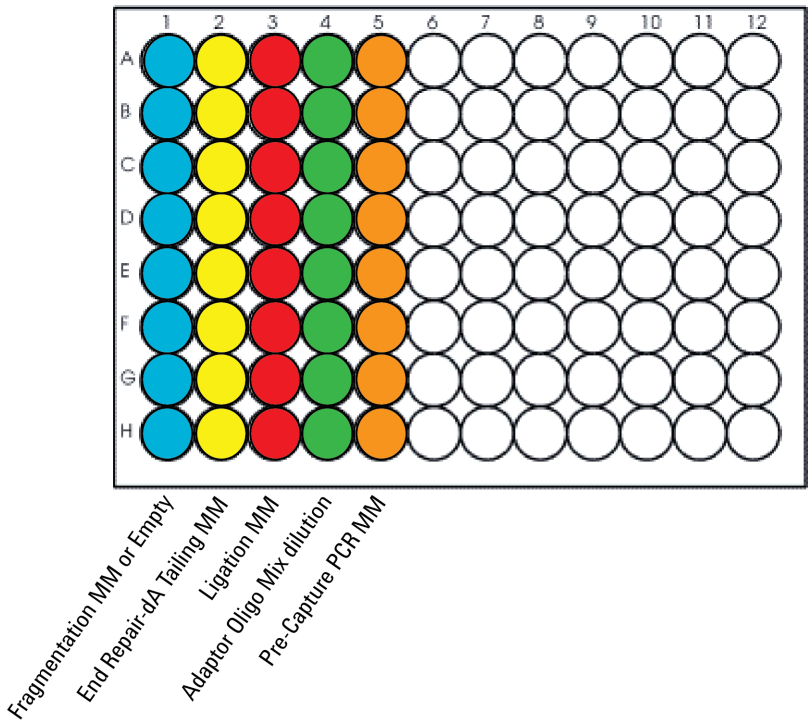


Figure 7 Configuration of the Agilent Deep Well master mix source plate for the library preparation runset. If you are using the enzymatic fragmentation workflow, column 1 contains fragmentation master mix. If you are using the mechanical fragmentation workflow, column 1 is empty.

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

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

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

Load the NGS Workstation

1 Load the Labware MiniHub according to **Table 30**, using the plate orientations shown in **Figure 8**.

The Labware MiniHub configuration shown in **Table 30** applies to both library preparation runsets (**EnzymaticFrag_LibPrep_XT_HS2** and **MechanicalFrag_LibPrep_XT_HS2**).

Table 30 Initial MiniHub configuration for the library preparation runsets

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Library preparation bead plate from **page 40** (80 µL of AMPure XP beads/well)	—	SureSelect XT HS2 Index Primer Pairs in processing plate with lid	Pre-capture PCR preparation bead plate from **page 42** (50 µL of AMPure XP beads/well)
Shelf 4	Empty processing plate	Empty processing plate	—	—
Shelf 3	Empty processing plate (no lid)	Empty processing plate (with lid, if Collect Sample option is not marked)	—	—
Shelf 2	New tip box	Nuclease-free water reservoir from **step 4**	Empty tip box	—
Shelf 1 (Bottom)	Empty tip box	70% ethanol reservoir with lid from **page 56**	Empty waste plate (Agilent 2 mL square well)	Empty tip box

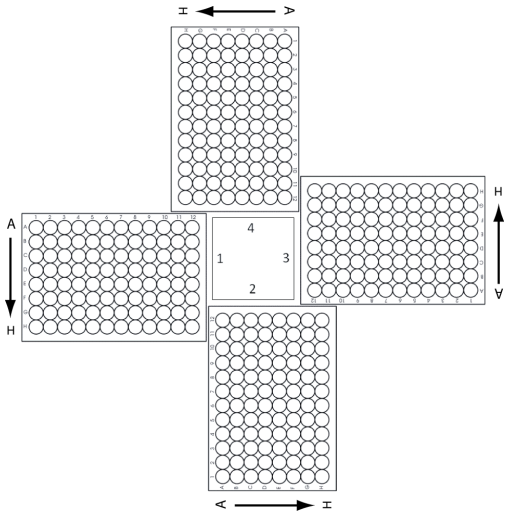


Figure 8 Agilent Labware MiniHub plate orientation.

2 Load the Bravo deck according to the appropriate table below.

- Enzymatic fragmentation workflow: Load the Bravo deck according to **Table 31**.
- Mechanical fragmentation workflow: Load the Bravo deck according to **Table 32**.

Table 31 Initial Bravo deck configuration for runset EnzymaticFrag_LibPrep_XT_HS2

Location	Content
4	Empty processing plate
6	DNA sample plate with lid prepared in **step 3** on **page 65**
9	Agilent deep well master mix source plate, unsealed

Table 32 Initial Bravo deck configuration for runset MechanicalFrag_LibPrep_XT_HS2

Location	Content
4	Sheared DNA sample plate (no lid)
9	Master mix source plate, unsealed

3 Load the BenchCel Microplate Handling Workstation according to the appropriate table below.

- Enzymatic fragmentation workflow: Load the BenchCel according to **Table 33**.
- Mechanical fragmentation workflow: Load the BenchCel according to **Table 34**.

Table 33 Initial BenchCel configuration for runset EnzymaticFrag_LibPrep_XT_HS2

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	3 Tip boxes	—	—	—
2	4 Tip boxes	—	—	—
3	5 Tip boxes	—	—	—
4	6 Tip boxes	—	—	—
6	8 Tip boxes	—	—	—
12	11 Tip boxes	4 Tip boxes	—	—

Table 34 Initial BenchCel configuration for runset MechanicalFrag_LibPrep_XT_HS2

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	2 Tip boxes	—	—	—
2	3 Tip boxes	—	—	—
3	4 Tip boxes	—	—	—
4	5 Tip boxes	—	—	—
6	7 Tip boxes	—	—	—
12	11 Tip boxes	2 Tip boxes	—	—

Run the library preparation runset (EnzymaticFrag_LibPrep_XT_HS2 or MechanicalFrag_LibPrep_XT_HS2)

1 In the VWorks software, open the SureSelect XT HS2 DNA form for the NGS Workstation Option B+. See **"Opening the SureSelect XT HS2 DNA form"** on page 34.

2 Under **Runset & Column Selection**, complete the settings according to **Table 35**.

Table 35 Runset & Column Selection settings

Setting	Option to select
Select runset to execute	Select the appropriate library preparation runset for your workflow. • Enzymatic fragmentation: Select **EnzymaticFrag_LibPrep_XT_HS2**. • Mechanical fragmentation: Select **MechanicalFrag_LibPrep_XT_HS2**.
Select the number of columns of samples to process	Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
Select processing plate type	Select the type of 96-well PCR processing plate you are using (Eppendorf twin.tec or Armadillo).

3 On the Protocol Parameters tab, under **Library Preparation Options**, complete the settings according to **Table 36**.

Table 36 Library Preparation Options settings

Setting	Option to select
Fragmentation Time	Select between **15 minutes** and **25 minutes**. See **Table 37** for guidance. This setting is only required for the **EnzymaticFrag_LibPrep_XT_HS2** runset, which includes the steps for enzymatic fragmentation.
Pre-Cap Pooling	Mark the check box if you are using the pre-capture pooling workflow. Clear the check box if you are using the post-capture pooling workflow.
Pre-PCR Cycle Number	Select the number of PCR cycles for the pre-capture amplification step of the runset. See **Table 38** for guidance.

Table 37 Fragmentation Time based on sample type and NGS read length

NGS read length requirement	Target fragment size	Fragmentation Time	
		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

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

4 On the Protocol Parameters tab, under **Sample Collection Option**, make a selection for the Collect Sample check box.

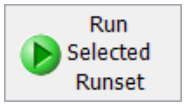
The EnzymaticFrag_LibPrep_XT_HS2 runset takes approximately 5 hours. The MechanicalFrag_LibPrep_XT_HS2 runset takes approximately 4 hours.

- Mark the check box if you plan to remove the sample plate from the NGS Workstation as soon as the runset is complete. The NGS Workstation leaves the sample plate uncovered and transfers it to Bravo deck position 7.
- Clear the check box if you may not be available to remove the sample plate from the NGS Workstation as soon as the runset is complete. The workstation covers the sample plate with a lid and holds it at 10°C on Bravo deck position 2.

5 Click **Display Initial Workstation Setup**.

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

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



At completion of the runset, the sample plate is located at position 7 of the Bravo deck.

8 Remove the plate from position 7 of the Bravo deck and seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing setting of 165°C and 1.0 seconds.

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

Clear the NGS Workstation of all plates and tip boxes and decontaminate the workstation surfaces according to the instructions in **"Decontamination Procedure"** on page 26.

Step 4. 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 TS_D1000) and perform analysis on Agilent 4200 TapeStation. See **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape"** on page 65.
- 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 70.

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

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

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

Prepare the NGS Workstation and Sample Buffer source plate

1. Turn off the ThermoCube device (see **page 21**) to restore position 9 of the Bravo deck to room temperature.
2. 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.
3. Using an Agilent deep well plate, prepare the D1000 Sample Buffer source plate at room temperature. Add the volume of D1000 Sample Buffer indicated in **Table 39** to each well of column 6 of the Agilent deep well plate.

Table 39 Preparation of the Sample Buffer source plate for TS_D1000 protocol

Solution	Position on Source Plate	Volume of D1000 Sample Buffer added per well of source plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
D1000 Sample Buffer	Column 6 (A6-H6)	11.0 µL	17.0 µL	23.0 µL	29.0 µL	42.0 µL	80.0 µL

CAUTION Make sure to add the D1000 Sample Buffer to column 6 of the source plate.

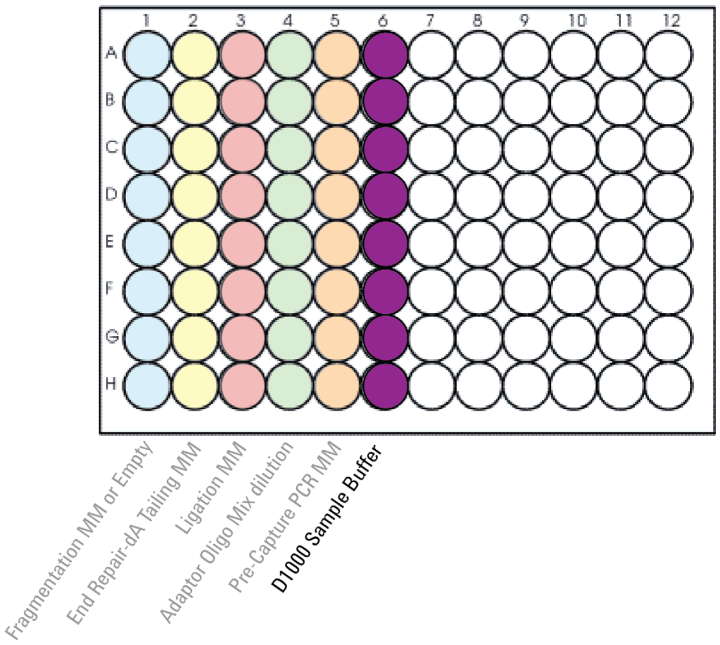


Figure 9 Configuration of the Agilent Deep Well source plate for protocol TS_D1000. The master mixes dispensed during the previous runset are shown in light shading (columns 1–5).

Load the NGS Workstation

4 Load the Labware MiniHub according to **Table 40**, using the plate orientations shown in **Figure 8** on page 61.

Table 40 Initial MiniHub configuration for TS_D1000 protocol

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

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

Table 41 Initial Bravo deck configuration for TS_D1000 protocol

Location	Content
4	Amplified pre-capture libraries in processing plate (unsealed)
6	Empty TapeStation analysis plate (Eppendorf twin.tec plate)
9	Agilent Deep Well source plate containing D1000 Sample Buffer in column 6

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

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

6 Load the BenchCel Microplate Handling Workstation according to **Table 42**.

Table 42 Initial BenchCel configuration for TS_D1000 protocol

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

Run VWorks protocol TS_D1000

7 In the VWorks software, open the Utility form. See **"Accessing the Supplemental Forms"** on page 36.

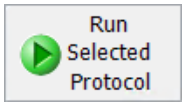
8 Under **Select protocol to execute**, select **TS_D1000**.

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

10 Click **Display Initial Workstation Setup**.

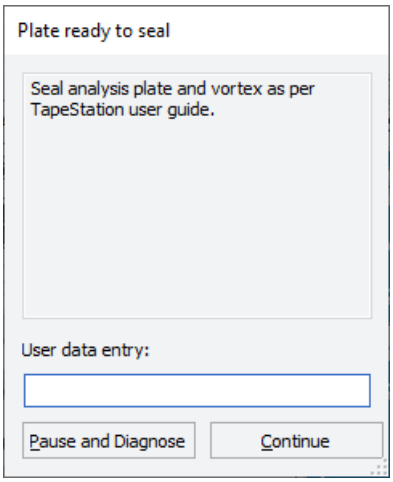
11 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



Running the TS_D1000 protocol takes approximately 15 minutes. Once removal of analytical samples is complete, remove the primary DNA library sample plate from position 4, seal the plate, and keep on ice until the samples are used for hybridization set up on **page 73**.

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 43** for guidelines). Sample electropherograms are shown in **Figure 10** (library prepared from high-quality DNA), **Figure 11** (library prepared from medium-quality FFPE DNA), and **Figure 12** (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 12**. See Troubleshooting information on **page 138** for additional considerations.

Table 43 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.

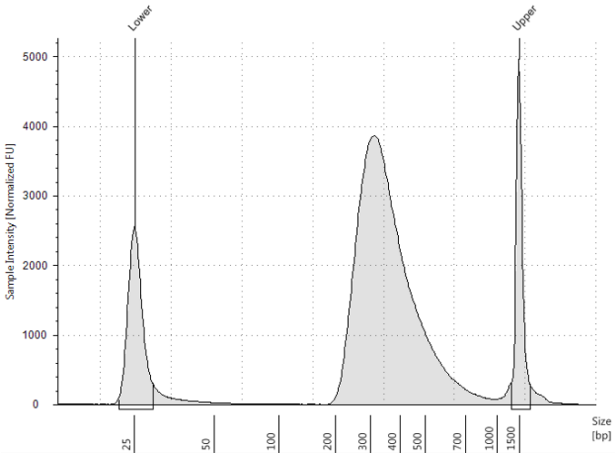


Figure 10 Pre-capture library prepared from a high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

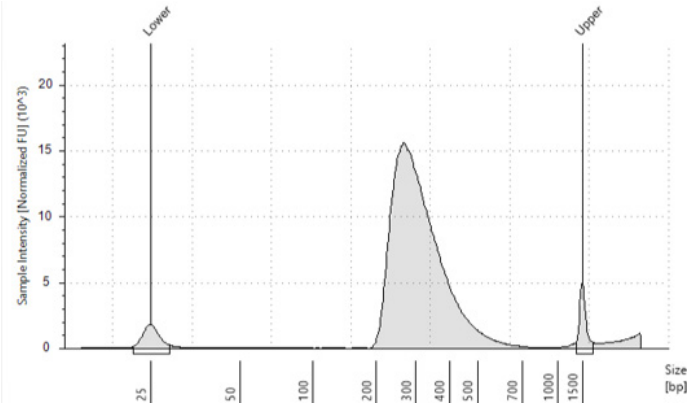


Figure 11 Pre-capture library prepared from a typical FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

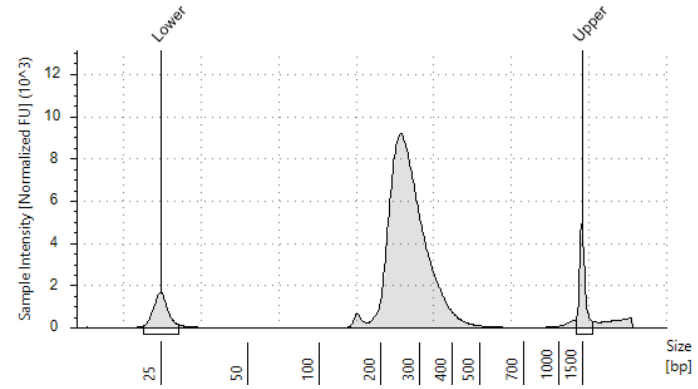


Figure 12 Pre-capture library prepared from a low-quality FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

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 10** through **Figure 12**). **Table 44**. Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 43** for guidelines). **Table 44** includes links to assay instruction.

Table 44 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.

6 Target Enrichment

This chapter provides instructions to complete the target enrichment steps, including hybridization and capture using a SureSelect Probe.

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 **"Step 1, Option 1. Prepare DNA for Single-Plex Hybridization"** on page 72.
- If you are pooling samples prior to hybridization to the Probe, then follow the steps in **"Step 1, Option 2. Prepare DNA for Multi-Plex Hybridization"** on page 75.

CAUTION The ratio of Probe to prepped library is critical for successful capture. Carefully follow the instructions provided to ensure you are using an optimal ratio.

Step 1, Option 1. Prepare DNA for Single-Plex Hybridization

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 **"Step 1, Option 2. Prepare DNA for Multi-Plex Hybridization"** on page 75.

Aliquot prepped DNA samples for hybridization

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.

Using the DNA concentration for each sample determined on **page 65** to **page 70**, 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 Aliquot_Libraries is used to prepare a new sample plate containing the appropriate quantity of each DNA sample in a 12-µL volume for hybridization. Before running the automation protocol, you must create a CSV (comma separated value) file containing instructions for the NGS Workstation indicating the volume of each sample to aliquot, as described in the steps below.

Create the CSV file for protocol Aliquot_Libraries

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

	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 13 Example CSV file content for the Aliquot_Libraries protocol

NOTE

The directory **C:\VWorks Workspace\NGS Option B Plus\XT_HS2_ILM_v.B1.0.3\Aliquot Input File Templates** contains an example CSV file for the Aliquot_Libraries protocol called *Aliquot_Libraries_Template.csv*.

You can copy this file and use it as a template for creating the CSV files for each Aliquot_Libraries protocol run. If you use this file as a template for runs with fewer than 12 columns, be sure to delete rows for any unused columns on the plate.

2 Enter the information requested in the header for each DNA sample.
   - In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
   - In the SourceWell and DestinationWell fields, enter each well position for the plate. SourceWell and DestinationWell field contents must be identical for a given sample.
   - In the Volume field, enter the volume (in µL) of each DNA sample to be used in the hybridization step (see **page 72** for guidelines). For all empty wells on the plate, delete the corresponding rows in the CSV file.
3 Load the CSV file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B Plus\XT_HS2_ILM_v.B1.0.3\Aliquot Input File Templates** or any desired directory on the local drive (do not save to OpenLab).

Prepare and load the NGS Workstation

4 Turn on the chiller, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
5 Load the Bravo deck according to **Table 45**.

Table 45 Initial Bravo deck configuration for Aliquot_Libraries protocol

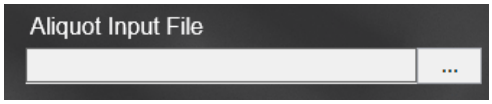
Location	Content
4	Nuclease-free water in Agilent shallow well reservoir (30 mL)
5	Empty tip box
6	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
8	New tip box
9	Prepped library DNA in processing plate

Run VWorks protocol Aliquot_Libraries

6 In the VWorks software, open the Utility form. See **"Accessing the Supplemental Forms"** on page 36.
7 Under **Select protocol to execute**, select the **Aliquot_Libraries** protocol.
8 Click **Display Initial Workstation Setup**.

9 Upload the CSV file created on **page 72**.

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

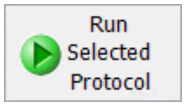


b Browse to the location where you saved the CSV file. Select the file and click **Open**.

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

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

11 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 6.

12 Remove the sample plate from the Bravo deck.

Continue to **"Step 2. Perform Hybridization and Capture"** on page 85.

Step 1, Option 2. Prepare DNA for Multi-Plex Hybridization

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 **"Step 1, Option 1. Prepare DNA for Single-Plex Hybridization"** on page 72.

NOTE

Preparing DNA for multi-plex hybridization includes pooling indexed DNA libraries into pools containing either 8 or 16 individual libraries. The process includes three separate automation protocols, performed in the following order.

- Aliquot_Water protocol - Adds water to the destination plate that will be used during the XT_HS2_Pooling protocol. The volume of water added to each well is enough to bring the pool volume to 100 µL after execution of the XT_HS2_ Pooling protocol. The Aliquot_Water protocol requires a CSV file listing the volume of water to be added to each well. Calculating the water volumes requires calculating the volumes of the library pools.
- XT_HS2_Pooling protocol - Adds individual indexed DNA libraries to the destination plate, creating library pools of 100 µL. The pooling protocol requires a CSV file for each source plate listing the specific wells to be pooled and the concentration of each sample.
- AMPureXP_XT_HS2_ILM protocol - Uses AMPure XP beads to purify the library pools, eluting them into 24 µL volumes.

Plan pooling cofigurations

Plan pooling run parameters

The hybridization reaction requires either 1500 ng or 3000 ng of indexed gDNA pool, depending on the Probe. The pool contains equal amounts of either 8 or 16 individual indexed gDNA libraries. See **Table 46** for the recommended pool composition based on your SureSelect Probe.

Table 46 Pre-capture pooling of indexed DNA libraries

Probe	Amount of total DNA per pool (Amount of DNA pool per hybridization reaction)*	Number of DNA libraries per pool	Maximum DNA concentration for pool	Amount of each DNA library in pool
SureSelect XT HS PreCap Human All Exon V8	6000 ng (3000 ng/hybridization)	8	375 ng/µL	750 ng
SureSelect XT HS PreCap Human All Exon V8+UTR	6000 ng (3000 ng/hybridization)	8	375 ng/µL	750 ng
SureSelect XT HS PreCap Human All Exon V8+NCV	6000 ng (3000 ng/hybridization)	8	375 ng/µL	750 ng
SureSelect XT HS PreCap Clinical Research Exome V4	6000 ng (3000 ng/hybridization)	8	375 ng/µL	750 ng
SureSelect Custom Probe	3000 ng (1500 ng/hybridization)	16	93.75 ng/µL	187.5 ng

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

Before pooling the DNA samples, 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. This process ensures that each individual library is equally represented in the pool.

Accurate pooling 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 46**. 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 46**, then prepare pools with either 3000 ng of DNA or 6000 ng of DNA, depending on the quantity required for your Probe (see the 2nd column in **Table 46**).
  - If at least one of the samples is above the maximum DNA concentration shown in **Table 46**, 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 configuration of the destination plate

During the automated pooling protocol, the required volumes of the indexed gDNA samples are transferred from the source plate(s) to the destination plate, where they are combined into pools of 8 or 16. The indexed gDNA samples should be pooled into the destination plate using a pooled sample configuration appropriate for the subsequent hybridization run. Consider the following guidelines when planning the configuration of the destination plate:

- 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. Place samples to be enriched using the same Probe in the same row.
- A single 96-reaction library preparation run produces 6 or 12 gDNA pools. For greatest efficiency of reagent use, you can set up the automated pooling protocol with multiple source plates (i.e., multiple library preparation runs) that consolidate into a single destination plate during pooling. This requires creating a separate CSV file for each source plate. A maximum of 16 source plates is possible (if each 96-well source plate yields 6 gDNA pools).

Prepare the destination plate for the library pools

During the automated pooling protocol, the NGS Workstation transfers to the appropriate volume of each individual DNA library to a well on the destination plate. To ensure that all library pools are normalized to the same 100-µL volume, the wells of the destination plate contain the required volume of water needed to bring the pool volume up to 100 µL. The Aliquot_Water automation protocol is used to prepare that destination plate. This protocol aliquots the required volume of water into the wells of the destination plate based on the calculated volume of each library pool. The destination plate is then used in the Pooling automation protocol.

Calculate the volume of each DNA library pool

During the automated pooling protocol, the NGS Workstation calculates the volume of each DNA library to add to the pool. This calculation is based on the following formula, where the *ng of DNA library in pool* value is either 750 ng, 187.5 ng, or 375 ng, depending on the Probe and number of individual DNA libraries per pool (as described in **Table 46** on page 75).

$$\mu L \text{ of DNA library added to pool} = \frac{\text{ng of DNA library in pool}}{\text{concentration of DNA library (in ng/}\mu L)}$$

Use this formula to calculate the volume of each individual DNA library in each DNA library pool. Then, use those values to calculate the total volume of each DNA library pool.

Prepare CSV file for normalizing library pool volumes to 100 µL

1 Create a CSV (comma separated value) file with the headers shown in **Figure 14**. 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 (**.csv*). The file must include rows for all 96 wells of the plate.

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

- In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
- In the SourceWell and DestinationWell fields, enter each well position for the plate. SourceWell and DestinationWell field contents must be identical for a given sample.
- In the Volume field, enter the volume (in µL) of water to be added to the indicated well position in order for the total well volume to be 100 µL after the DNA libraries are added to the well. 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 14 Example CSV file content for the Aliquot_Water protocol

NOTE

The directory **C:\VWorks Workspace\NGS Option B Plus\XT_HS2_ILM_v.B1.0.3\Aliquot Input File Templates** contains an example CSV file for the Aliquot_Water protocol called *Aliquot_Water_Template.csv*. You can copy this file and use it as a template for creating the CSV file. If you use this file as a template for runs with fewer than 12 columns, be sure to delete rows for any unused columns on the plate.

3 Load the CSV file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B Plus\XT_HS2_ILM_v.B1.0.3\Aliquot Input File Templates** or any desired directory on the local drive (do not save to OpenLab).

Prepare and load the NGS Workstation for protocol Aliquot_Water

4 Turn off the ThermoCube device (see **page 21**) to restore position 9 of the Bravo deck to room temperature.

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

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

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

Table 47 Initial Bravo deck configuration for Aliquot_Water protocol

Location	Content
5	Empty tip box
6	Empty processing plate
8	New tip box
9	Nuclease-free water reservoir from **step 5**

Run VWorks protocol Aliquot_Water

7 In the VWorks software, open the Utility form. See **"Accessing the Supplemental Forms"** on page 36.

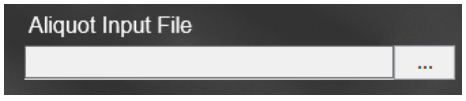
8 Under **Select protocol to execute**, select the **Aliquot_Water** protocol.

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

10 Click **Display Initial Workstation Setup**.

11 Upload the CSV file created on **page 77**.

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

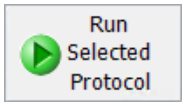


b Browse to the location where you saved the CSV file. Select the file and click **Open**.

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

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

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



The water aliquoting protocol takes approximately 1 hour for 96 sample pools. When complete, the destination plate is on Bravo deck position 6.

14 Remove the destination plate from the Bravo deck.

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

Pool indexed DNA libraries and purify library pools

In this step, the NGS Workstation uses the Pooling automation protocol to pool the prepped indexed gDNA libraries followed by the AMPureXP_XT_HS2_ILM (Case: Concentration of Pool) automation protocol to concentrate each DNA library pool to 24 µL, a volume sufficient for two hybridization reactions.

The Pooling automation protocol is set up and run from the VWorks Pooling and Normalization form (see **"Overview of the Pooling and Normalization form"** on page 37). The AMPureXP_XT_HS2_ILM (Case: Concentration of Pool) automation protocol is set up and run from the Utility form (see **"Accessing the Supplemental Forms"** on page 36).

Prepare CSV files for pooling

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

See **Figure 15** for required CSV file content. The directory **C:\VWorks Workspace\NGS Option B Plus\XT_HS2_ILM_v.B1.0.3\Pooling and Normalization Templates** contains CSV file templates for the automated pooling protocol. Select the appropriate set of templates from the directory based on the intended pool composition (8 or 16 prepared samples) and on the number of source plates to be consolidated in the run to prepare the single destination plate containing the hybridization samples. For example, for 8-library pools, use the template Pool8_01_SourcePlate.csv for the first DNA source plate, continuing with additional Pool8_0X_SourcePlate.csv files for additional DNA source plates.

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

   If processing a partial plate of prepped gDNA samples, delete the rows corresponding to the Well IDs of the empty wells on the source 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 15 Example of CSV file content for pooling protocol

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

- In the **PreCap Amplified pond concentrations** field, enter the concentration (in ng/µL) determined on **page 65** for each indexed DNA library. The automated protocol uses the concentration to calculate the required volume of DNA library to be added to the pool on the destination plate.
- In the **Target WellID** field, enter the well position on the destination plate where the required volume of the indexed DNA sample is to be transferred. This target well will contain the pooled gDNA samples. See the guidelines on **page 75** for hybridization sample pool placement considerations.

Prepare the NGS Workstation for the XT_HS2_Pooling protocol

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 Turn on the chiller, 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.

Run the VWorks XT_HS2_Pooling protocol

5 In the VWorks software, open the Pooling and Normalization form. See **"Accessing the Supplemental Forms"** on page 36.

6 In the Pooling and Normalization form, enter the run settings according to **Table 48**.

Figure 16 shows the sections of the form that contain the settings.

Table 48 Pooling run settings

Form section/Setting		Option to select
Pooling Options	Number of Indexes to Pool	Select 8 or 16 (see **Table 46** on page 75 for guidelines)
	Pooled DNA Quantity	Enter the required total amount of DNA in the pool. The required amount is either 3000 ng or 6000 ng, depending on the Probe (see **Table 46** on page 75). These amounts are sufficient for two hybridization reactions.
Destination Plate ID/Barcode		Enter the name or barcode of the destination plate into the field provided.
Processing Plate Type		Select the type of 96-well PCR processing plate you are using (Eppendorf twin.tec or Armadillo) for the source plate(s) and destination plate.

Table 48 Pooling run settings

Form section/Setting		Option to select
Source Plates	Number of Source Plates	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.
	Load Sources	Specify whether the indexed DNA source plate(s) will be loaded to the MiniHub (recommended) or loaded manually.
	Sources Enter Sealed?	Specify whether the indexed DNA source plate(s) will be sealed at start of run (recommended).
	Concentration File	Click the browse button ("...") to specify the location of each CSV file that provides sample position and concentration data for each plate.

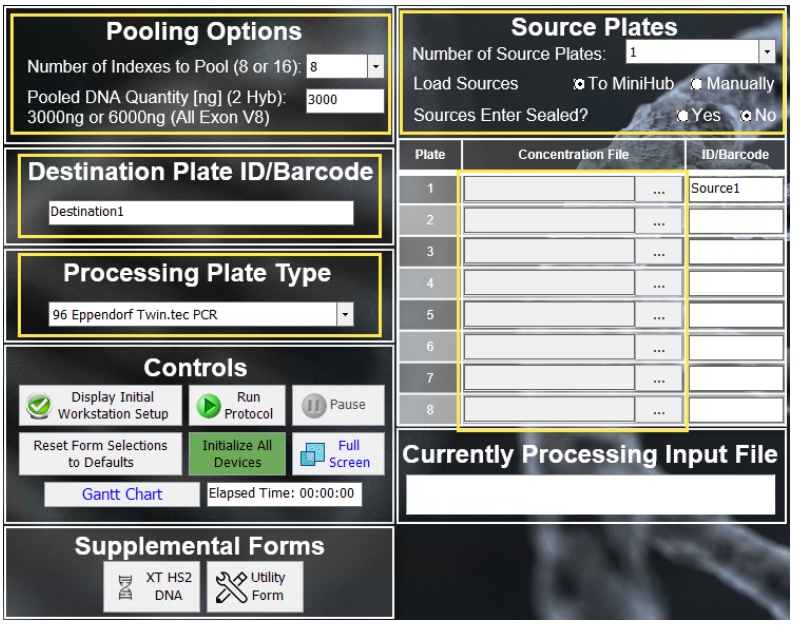


Figure 16 Pooling and Normalization form - sections for run settings

7 When finished selecting run parameters, click **Display Initial Workstation Setup**.

8 Load sample plates and labware as displayed on the right side of the form (example shown in **Figure 17** below is for pooling run with 8 source plates):

- Load each indexed DNA source plates onto its assigned shelf on the MiniHub (unless you selected manual loading of source plates)
- Load the destination plate that was prepared earlier (see **"Prepare the destination plate for the library pools"** on page 76) on Bravo deck position 5.
- Load an empty tip box on Bravo deck position 6.
- Load the indicated number of tip boxes in BenchCel Stacker 1.

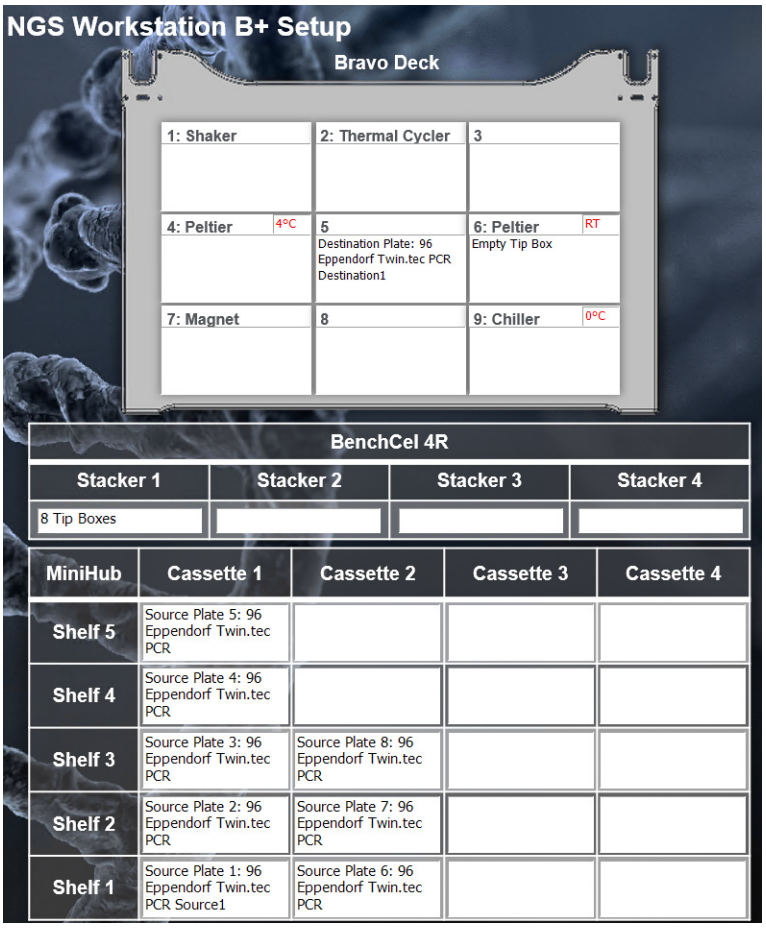
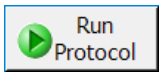


Figure 17 Example of NGS Workstation setup for pooling run with 8 source plates (Eppendorf twin.tec plates) loaded to the MiniHub

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



CAUTION When more than one source plate is used in the run, an operator must be present during the run to remove and replace plate seals during the run, in response to NGS Workstation prompts.

Running the pooling protocol takes approximately one hour per indexed DNA source plate. Once complete, the destination plate, containing indexed DNA pools in a 100-µL volume, is located at position 5 of the Bravo deck.

10 Remove the destination plate from Bravo deck position 5. This plate contains the indexed DNA library pools, normalized to 100 µL.

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

Prepare and load the NGS Workstation for protocol AMPureXP_XT_HS2_ILM (Case: Concentration of Pool)

The AMPureXP_XT_HS2_ILM protocol uses AMPure XP beads to purify the DNA library pools, eluting the DNA in a volume of 24 µL, which is sufficient for two hybridization reactions.

This step uses the aliquoted plate of AMPure XP beads that was prepared on **page 44**.

12 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.

13 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.

14 Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol. Add a lid to prevent evaporation.

15 Load the Labware MiniHub according to **Table 49**, using the plate orientations shown in **Figure 8** on page 61.

Table 49 Initial MiniHub configuration for AMPureXP_XT_HS2_ILM (Case: Concentration of Pool) protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Concentration of pooled DNA bead plate from **page 44** (180 µL of AMPure XP beads/well)	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	Empty processing Plate	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 13**		—
Shelf 1 (Bottom)	—	70% ethanol reservoir with lid from **step 14**	Empty waste plate (Agilent 2 mL square well)	Empty tip box

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

Table 50 Initial Bravo deck configuration for AMPureXP_XT_HS2_ILM (Case: Concentration of Pool) protocol

Location	Content
4	Destination plate from the XT_HS2_Pooling protocol containing 100-µL DNA library pools

17 Load the BenchCel Microplate Handling Workstation according to **Table 51**.

Table 51 Initial BenchCel configuration for AMPureXP_XT_HS2_ILM (Case: Concentration of Pool) protocol

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

Run VWorks protocol AMPure_XT_HS2_ILM (Case: Concentration of Pool)

18 In the VWorks software, open the Utility form. See **"Accessing the Supplemental Forms"** on page 36.

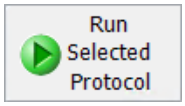
19 Under **Select protocol to execute**, select the **AMPureXP_XT_HS2_ILM (Case: Concentration of Pool)** protocol.

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

21 Click **Display Initial Workstation Setup**.

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

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



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

Continue to **"Step 2. Perform Hybridization and Capture"** on page 85.

Step 2. Perform Hybridization and Capture

In this step, automation runset **SSELHyb&Capture_XT_HS2** is used to execute the following hybridization and capture steps.

- Hybridization of the DNA libraries and Probe
- Capture of the hybridized DNA on magnetic beads
- Washing of the captured DNA
- Amplification of the captured libraries (post-capture PCR)
- Purification of the amplified indexed libraries

This step uses the components listed in **Table 52**. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 52 Reagents for Hybridization

Kit Component	Storage Location	Preparation Notes	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 87**
SureSelect RNase Block (purple cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	**page 88**
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 88**
Probe	–80°C	Thaw on ice	**page 88**
SureSelect Binding Buffer	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	—	**page 86**
SureSelect Wash Buffer 1	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	—	**page 86**
SureSelect Wash Buffer 2	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	—	**page 86**
SureSelect Streptavidin Beads or Dynabeads MyOne Streptavidin T1	SureSelect Streptavidin Beads (bottle) are stored at 4°C until just before use, or follow storage recommendations provided by supplier (see **Table 1** on page 11)	—	**page 86**
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Mix by pipetting up and down 15–20 times	**page 88**
5× Herculase II Reaction Buffer with dNTPs (clear cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Mix on a vortex	**page 88**
SureSelect Post-Capture Primer Mix (clear cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Mix on a vortex	**page 88**

Prepare the NGS Workstation and reagent reservoirs

1. Pre-set the temperature of Bravo deck positions 4 and 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 19.

   Bravo deck position 4 corresponds to CPAC 2, position 1. Bravo deck position 6 corresponds to CPAC 2, position 2.

2. Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water. Place the reservoir at Bravo deck position

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

3. Place the water reservoir on the MiniHub at shelf 2, cassette 2.
4. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol. Add a lid to prevent evaporation.
5. Place the 70% ethanol reservoir on the MiniHub at shelf 1, cassette 2.

Prepare wash solution source plates

1. 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. Add a lid to the plate.
2. Place the *Wash #1* plate on the MiniHub at shelf 4, cassette 2.
3. Prepare an Agilent Deep Well source plate labeled *Wash #2*. For each well to be processed, add 1150 µL of SureSelect Wash Buffer 2.
4. Place the *Wash #2* plate on the MiniHub at shelf 5, cassette 2.

Prepare the streptavidin beads

1. Vigorously resuspend the Streptavidin T1 magnetic beads on a vortex mixer. The beads settle during storage.
2. Wash the magnetic beads.
   a. In a conical vial, combine the components listed in **Table 53**. The volumes below include the required overage.

Table 53 Magnetic bead washing mixture

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Streptavidin T1 bead suspension	50 µL	425 µL	825 µL	1225 µL	1.65 mL	2.5 mL	5.0 mL
SureSelect Binding Buffer	0.2 mL	1.7 mL	3.3 mL	4.9 mL	6.6 mL	10 mL	20 mL
Total Volume	**0.25 mL**	**2.125 mL**	**4.125 mL**	**6.125 mL**	**8.25 mL**	**12.5 mL**	**25 mL**

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.)

3 Resuspend the beads in SureSelect Binding buffer, according to **Table 54** below.

Table 54 Preparation of magnetic beads for SSELHyb&Capture_XT_HS2 runset

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Binding Buffer	0.2 mL	1.7 mL	3.3 mL	4.9 mL	6.6 mL	10 mL	20 mL

4 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.

5 Place the streptavidin bead source plate on the MiniHub at shelf 5, cassette 1.

Prepare the Block master mix

1 Prepare the appropriate volume of Block master mix, on ice, as indicated in **Table 55**. Mix by vortexing at medium speed for 15–20 seconds. Keep on ice.

Table 55 Preparation of Block master mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2.5 µL	31.9 µL	53.1 µL	74.4 µL	95.6 µL	138.1 µL	276.3 µL
SureSelect XT HS2 Blocker Mix (blue cap)	5.0 µL	63.8 µL	106.3 µL	148.8 µL	191.3 µL	276.3 µL	552.5 µL
Total Volume	**7.5 µL**	**95.7 µL**	**159.4 µL**	**223.2 µL**	**286.9 µL**	**414.4 µL**	**828.8 µL**

Prepare the post-capture PCR master mix

1 Prepare the appropriate volume of post-capture PCR Master Mix, according to **Table 56**. Mix well using a vortex mixer and keep on ice.

Table 56 Preparation of Post-Capture PCR Master Mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
5× Herculase II Reaction Buffer with dNTPs (clear cap)	10 µL	170 µL	255 µL	340 µL	425 µL	574 µL	1105µL
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	17.0 µL	25.5 µL	34.0 µL	42.5 µL	57.4 µL	110.5 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17.0 µL	25.5 µL	34.0 µL	42.5 µL	57.4 µL	110.5 µL
Total Volume	**12.0 µL**	**204.0 µL**	**306.0 µL**	**408.0 µL**	**510.0 µL**	**688.8 µL**	**1326.0 µL**

Prepare one or more Probe Hybridization master mixes

1 Prepare the appropriate volume of Probe Hybridization master mix for each of the Probes that will be used for hybridization as indicated in **Table 57** to **Table 60**. 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 57** or **Table 58**) on **page 88**.

For runs that use different Probes for individual rows, prepare each master mix as described in Step b (**Table 59** or **Table 60**) on **page 89**.

a For runs that use a single Probe for all rows, prepare a master mix as described in **Table 57** or **Table 58**, according to the Probe design size.

Table 57 Preparation of Probe Hybridization master mix for Probes <3 Mb, 8 rows of wells

	Target size <3.0 Mb						
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	7.0 µL	89.3 µL	148.8 µL	208.3 µL	267.8 µL	401.6 µL	818.1 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	28.7 µL	58.4 µL
SureSelect Fast Hybridization Buffer (bottle)	6.0 µL	76.5 µL	127.5 µL	178.5 µL	229.5 µL	344.3 µL	701.3 µL

Table 57 Preparation of Probe Hybridization master mix for Probes <3 Mb, 8 rows of wells

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Probe (with design <3.0 Mb)	2.0 µL	25.5 µL	42.5 µL	59.5 µL	76.5 µL	114.8 µL	233.8 µL
Total Volume	**15.5 µL**	**197.6 µL**	**329.4 µL**	**461.1 µL**	**592.9 µL**	**889.4 µL**	**1811.6 µL**

Table 58 Preparation of Probe Hybridization master mix for Probes ≥3 Mb, 8 rows of wells

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.0 µL	51.0 µL	85.0 µL	119.0 µL	153.0 µL	229.5 µL	467.5 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	28.7 µL	58.4 µL
SureSelect Fast Hybridization Buffer (bottle)	6.0 µL	76.5 µL	127.5 µL	178.5 µL	229.5 µL	344.3 µL	701.3 µL
Probe (with design ≥3.0 Mb)	5.0 µL	63.8 µL	106.3 µL	148.8 µL	191.3 µL	286.9 µL	584.4 µL
Total Volume	**15.5 µL**	**197.6 µL**	**329.4 µL**	**461.1 µL**	**592.9 µL**	**889.4 µL**	**1811.6 µL**

b For runs that use different Probes in individual rows, prepare a master mix for each Probe as listed in **Table 59** or **Table 60**, according to the Probe design size. The volumes listed in **Table 59** and **Table 60** 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.

All Probes used in the same run need to use the same post-capture PCR cycle number. Refer to **Table 66** for cycle number requirements based on Probe size.

Table 59 Preparation of Probe Hybridization master mix for Probes <3 Mb, single row of wells

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	7 µL	10.5 µL	17.5 µL	24.5 µL	31.5 µL	49.0 µL	98.0 µL
RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.3 µL	3.5 µL	7.0 µL
SureSelect Fast Hybridization Buffer (bottle)	6.0 µL	9.0 µL	15.0 µL	21.0 µL	27.0 µL	42.0 µL	84.0 µL
Probe (with design <3 Mb)	2.0 µL	3.0 µL	5.0 µL	7.0 µL	9.0 µL	14.0 µL	28.0 µL
Total Volume	**15.5 µL**	**23.3 µL**	**38.8 µL**	**54.3 µL**	**69.8 µL**	**108.5 µL**	**217.0 µL**

Table 60 Preparation of Probe Hybridization master mix for Probes ≥3 Mb, single row of wells

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.0 µL	6.0 µL	10.0 µL	14.0 µL	18.0 µL	28.0 µL	56.0 µL
RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.3 µL	3.5 µL	7.0 µL
SureSelect Fast Hybridization Buffer (bottle)	6.0 µL	9.0 µL	15.0 µL	21.0 µL	27.0 µL	42.0 µL	84.0 µL
Probe (with design ≥3 Mb)	5.0 µL	7.5 µL	12.5 µL	17.5 µL	22.5 µL	35.0 µL	70.0 µL
Total Volume	**15.5 µL**	**23.3 µL**	**38.8 µL**	**54.3 µL**	**69.8 µL**	**108.5 µL**	**217.0 µL**

Prepare the master mix source plate

1 Using a processing plate, prepare the hybridization master mix source plate at room temperature. The source plate contains the Block master mix (**Table 55**), the Post-Capture PCR master mix (**Table 56**), and the Probe Hybridization master mix (**Table 57** through **Table 60**). Add the volumes indicated in **Table 61** of each master mix to each well of the indicated column of the plate. When using multiple Probes in a run, add the Probe Hybridization master mix for each Probe to the appropriate row(s) of the plate. The final configuration of the master mix source plate is shown in **Figure 18**.

Table 61 Preparation of the master mix source plate for SSELHyb&Capture_XT_HS2 runset

Master Mix Solution	**Position on Source Plate**	**Volume of master mix added per Well of Processing Source Plate**					
		1-Column Runs	**2-Column Runs**	**3-Column Runs**	**4-Column Runs**	**6-Column Runs**	**12-Column Runs**
Block master mix	Column 1 (A1-H1)	11.0 µL	19.0 µL	27.0 µL	34.9 µL	50.9 µL	102.7 µL
Probe Hybridization master mix	Column 2 (A2-H2)	23.3 µL	38.8 µL	54.3 µL	69.8 µL	108.5µL	217.0 µL*
Post-Capture PCR master mix	Column 3 (A3-H3)	23.0 µL	36.0 µL	49.0 µL	62.0 µL	82.0 µL	163.0 µL

* 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.

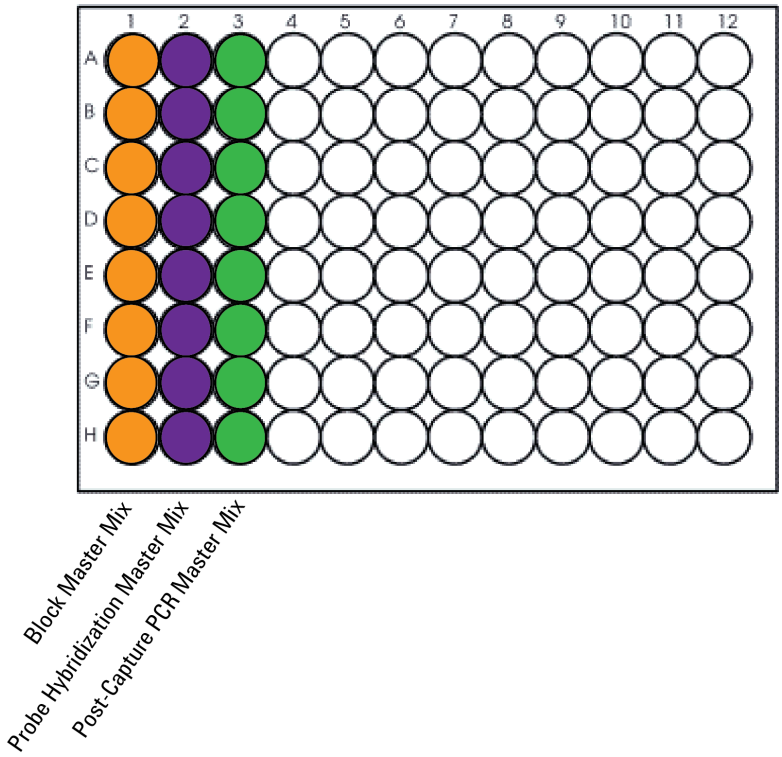


Figure 18 Configuration of the master mix source plate for runset SSELHyb&Capture_XT_HS2. Column 2 can contain different Probe Hybridization master mixes in each row.

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

Load the NGS Workstation

1 Load the Labware MiniHub according to **Table 62**, using the plate orientations shown in **Figure 8** on page 61.

Table 62 Initial MiniHub configuration for the SSELHyb&Capture_XT_HS2 runset

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Streptavidin bead Deep Well source plate	*Wash #2* Deep Well source plate	Empty processing plate with lid (used for post-capture PCR reactions)	Post-capture PCR bead plate with lid from **page 46** (50 µL of AMPure XP beads/well)
Shelf 4		*Wash #1* processing source plate with lid		
Shelf 3		Empty processing plate (with lid, if Collect Sample option is not marked) to be used as collection plate		
Shelf 2		Nuclease-free water reservoir		
Shelf 1 (Bottom)	Empty tip box	70% ethanol reservoir with lid	Empty waste plate (Agilent 2 mL square well)	Empty tip box

2 Load the Bravo deck according to **Table 63**.

Table 63 Initial Bravo deck configuration for the SSELHyb&Capture_XT_HS2 runset

Location	Content
1	Empty processing plate with lid (Eppendorf twin.tec or Armadillo plate)
4	Master Mix source plate (unsealed)
6	Prepared library aliquots or library pools in processing plate (unsealed)
7	Empty processing plate (Eppendorf twin.tec or Armadillo plate)

3 Load the BenchCel Microplate Handling Workstation according to **Table 64**.

Table 64 Initial BenchCel configuration for the SSELHyb&Capture_XT_HS2 runset

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	2 Tip boxes	—	—	—
2	3 Tip boxes	—	—	—
3	4 Tip boxes	—	—	—
4	5 Tip boxes	—	—	—
6	7 Tip boxes	—	—	—
12	11 Tip boxes	2 Tip boxes	—	—

Run VWorks runset SSELHyb&Capture_XT_HS2

1 In the VWorks software, open the SureSelect XT HS2 DNA form for the NGS Workstation Option B+. See **"Opening the SureSelect XT HS2 DNA form"** on page 34.

2 Under **Runset & Column Selection**, complete the settings according to **Table 56**.

Table 65 Runset & Column Selection settings

Setting	Option to select
Select runset to execute	Select **SSELHyb&Capture_XT_HS2**
Select the number of columns of samples to process	Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
Select processing plate type	Select the type of 96-well PCR processing plate you are using (Eppendorf twin.tec or Armadillo).

3 On the Protocol Parameters tab, under **Target Enrichment Options**, complete the settings according to **Table 66**.

Table 66 Target Enrichment Options settings

Setting	Option to select
Hyb Extension	Agilent recommends clearing the Hyb Extension check box unless previous data with your Probe(s) indicate that an extended hybridization on the thermal cycler may improve performance.* See **page 138** in the **"Troubleshooting Guide"** for more information.
Post-PCR Cycle Number	Select the number of PCR cycles for the post-capture PCR amplification step of the runset. The recommended number of cycles depends on the size of the Probe. 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

* Recommended settings for Target Enrichment Options were established using representative Probes, e.g., SureSelect XT HS Human All Exon V8.

4 On the Protocol Parameters tab, under **Sample Collection Option**, make a selection for the Collect Sample check box.

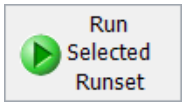
The target enrichment runset takes approximately 6.5 to 7 hours.

- Mark the check box if you plan to remove the sample plate from the NGS Workstation as soon as the runset is complete. The workstation leaves the sample plate uncovered and transfers it to Bravo deck position 7.
- Clear the check box if you may not be available to remove the sample plate from the NGS Workstation as soon as the runset is complete. The workstation covers the sample plate with a lid and holds it at 10°C on Bravo deck position 2.

5 Click **Display Initial Workstation Setup**.

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

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



If you pooled the DNA libraries for multi-plex hybridization, then the processing plate containing the library pools contains enough of each pool for two hybridization reactions. Once the BenchCel transfers that processing plate with the remaining library pools to the MiniHub (cassette 1, shelf 4), you can carefully retrieve it from the MiniHub (do not pause the runset), seal it, and store it at –20°C in the event that the remaining samples require further processing.

When complete, the amplified, purified DNA libraries or library pools are in the processing plate located on Bravo deck position 7.

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 an Agilent 4200 TapeStation Instrument and High Sensitivity D1000 ScreenTape"** on page 95.
- 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 100.

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

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

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

Prepare the NGS Workstation and Sample Buffer source plate

1 Turn off the ThermoCube device (see **page 21**) to restore position 9 of the Bravo deck to room temperature.

2 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.

3 Using the same master mix source plate (Eppendorf twin.tec or Armadillo plate) that was used for the SSELHyb&Capture_XT_HS2 runset, prepare the Sample Buffer source plate at room temperature. Add the volume of High Sensitivity D1000 Sample Buffer indicated in **Table 61** to each well of column 4 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 19**.

Table 67 Preparation of the Sample Buffer Source Plate for TS_HighSensitivity_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Processing Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
High Sensitivity D1000 Sample Buffer	Column 4 (A4-H4)	8.0 µL	11.0 µL	14.0 µL	17.0 µL	24.0 µL	44.0 µL

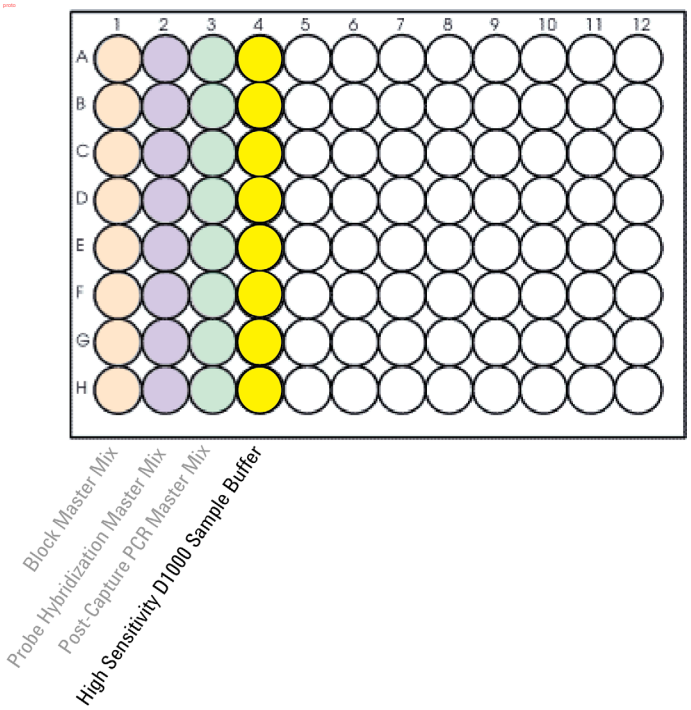


Figure 19 Configuration of the processing source plate for protocol TS_High-Sensitivity_D1000. Columns 1–3 were used to dispense master mixes during the previous runset.

Load the NGS Workstation

4 Load the Labware MiniHub according to **Table 68**, using the plate orientations shown in **Figure 8** on page 61.

Table 68 Initial MiniHub configuration for TS_HighSensitivity_D1000 protocol

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

5 Load the Bravo deck according to **Table 69**.

Table 69 Initial Bravo deck configuration for TS_HighSensitivity_D1000 protocol

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

CAUTION

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

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

6 Load the BenchCel Microplate Handling Workstation according to **Table 70**.

Table 70 Initial BenchCel configuration for TS_HighSensitivity_D1000 protocol

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

Run VWorks protocol TS_HighSensitivity_D1000

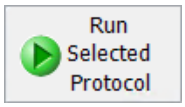
7 In the VWorks software, open the Utility form. See **"Accessing the Supplemental Forms"** on page 36.

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

9 Click **Display Initial Workstation Setup**.

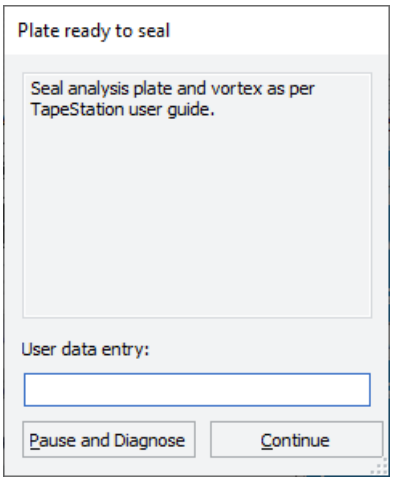
10 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



Running the TS_HighSensitivity_D1000 protocol takes approximately 15 minutes. Once removal of analytical samples is complete, remove the primary DNA library sample plate from position 4, seal the plate, and keep on ice until the samples are pooled for sequencing on **page 102**.

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



CAUTION

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

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

Run the High Sensitivity D1000 Assay and analyze the data

13 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.

14 Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 71** for guidelines). Sample electropherograms are shown in **Figure 20** (library prepared from high-quality DNA), **Figure 21** (library prepared from medium-quality FFPE DNA), and **Figure 22** (library prepared from low-quality FFPE DNA).

Table 71 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

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

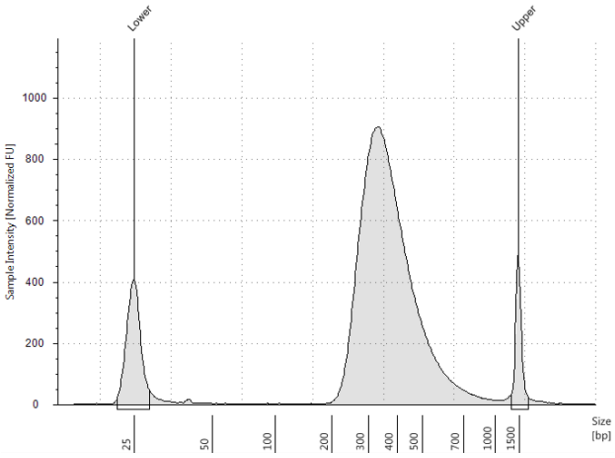


Figure 20 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

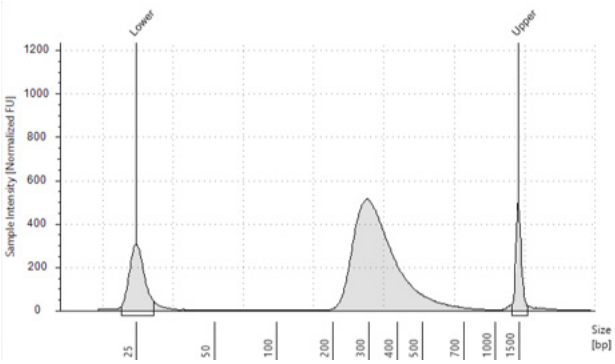


Figure 21 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

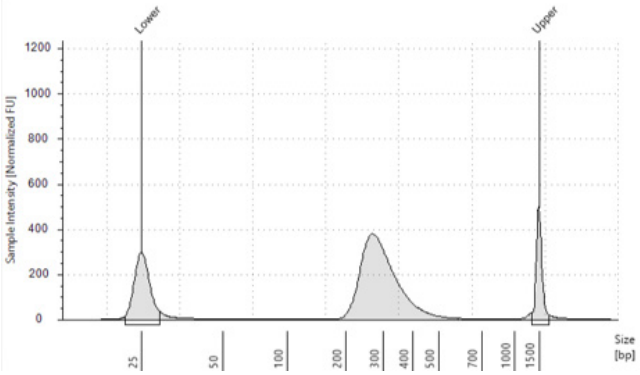


Figure 22 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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 20** through **Figure 22**). Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 71** for guidelines). **Table 72** includes links to assay instructions.

Table 72 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.

7 Sequencing Preparation and Processing

This chapter provides instructions for preparing samples for sequencing, guidelines for performing the sequencing, and information on sequence data analysis.

Step 1. 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 2. Prepare sequencing samples"** on page 106.

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 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 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. Use the formula below to determine 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 73 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 73 Example of volume calculation for total volume of 20 µL at 10 nM concentration

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

Pool samples for multiplexed sequencing using automation (optional for Method 2)

The instructions below are for **Method 2**. Alternatively, you can perform this method using an entirely manual approach rather than with the Aliquot_Captures automation protocol.

1 Create a CSV (comma separated value) file with the headers shown in **Figure 23**. 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 23** 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 23 Example of CSV file content for Aliquot_Captures protocol

NOTE

The directory **C:\VWorks Workspace\NGS Option B Plus\XT_HS2_ILM_v.B1.0.3\Aliquot Input File Templates** contains an example CSV file for the Aliquot_Captures protocol called *Aliquot_Captures_template.csv*. You can copy this file and use it as a template for creating the CSV file. If you are using the sample file as a template for runs with fewer than 12 columns, be sure to delete rows for any unused columns on the plate.

3 Load the CSV file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B Plus\XT_HS2_ILM_v.B1.0.3\Aliquot Input File Templates** or any desired directory on the local drive (do not save to OpenLab).

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

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

Table 74 Initial Bravo deck configuration for Aliquot_Captures protocol

Location	Content
5	Empty tip box
6	Empty Agilent deep well plate
8	New tip box
9	Purified amplified indexed libraries in processing plate

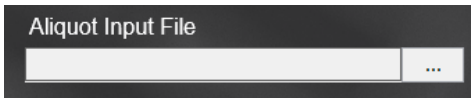
6 In the VWorks software, open the Utility form. See **"Accessing the Supplemental Forms"** on page 36.

7 Under **Select protocol to execute**, select the **Aliquot_Captures** protocol.

8 Click **Display Initial Workstation Setup**.

9 Upload the CSV file created in **step 1** through **step 3** (page **103** to **104**).

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

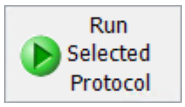


b Browse to the location where you saved the CSV file. Select the file and click **Open**.

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

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

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



The aliquoting protocol takes approximately 1 hour for 96 samples. When complete, the destination plate containing the library pools is on Bravo deck position 6.

12 Remove the destination plate from the Bravo deck.

13 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 2. 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 24**.

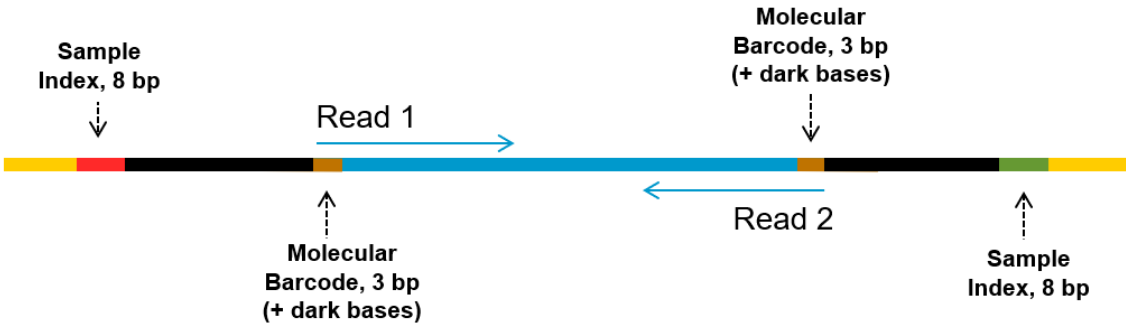


Figure 24 Content of SureSelect XT HS2 sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the dual sample indexes (red and green), molecular barcodes (brown) and the library PCR primers (yellow).

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. **Table 75** provides guidelines for use of several instrument and chemistry combinations suitable for this application. For other Illumina NGS platforms, consult Illumina's documentation for kit configuration and seeding concentration guidelines.

Table 75 Illumina Kit Configuration Selection Guidelines

Platform	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
MiSeq	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	9–10 pM
MiSeq	All Runs	2 × 75 bp	150 Cycle Kit	v3	12–16 pM
iSeq 100	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	50–150 pM
NextSeq 500/550	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2.5	1.2–1.5 pM
NextSeq 1000/2000	All Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1, v2, or v3	650–1000 pM
HiSeq 000	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	200 or 300 Cycle Kit	v1.0 or v1.5	300–600 pM
NovaSeq 6000	Xp Workflow Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1.0 or v1.5	200–400 pM
NovaSeq X	All Runs	2 × 100 bp or 2 × 150 bp	100. 200, or 300 Cycle Kit	v1	90–180 pM

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 75** or provided by Illumina. Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Step 3. Sequence the libraries

Set up the sequencing run to generate Read 1 and Read 2 FASTQ files for each sample using the instrument's software in standalone mode or using an Illumina run management tool such as Local Run Manager (LRM), Illumina Experiment Manager (IEM) or BaseSpace. Enter the appropriate **Cycles** or **Read Length** value for your library read length and using 8-bp dual index reads. See **Table 76** showing example settings for 2x150 bp sequencing..

Table 76 Run settings

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

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

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

- Each of the sample-level indexes (i7 and i5) requires an 8-bp index read. For complete index sequence information, see **Table 87** on page 121 through **Table 94** on page 128.
- No custom primers are used for SureSelect XT HS2 library sequencing. Leave all *Custom Primers* options for *Read 1, Read 2, Index 1* and *Index 2* primers cleared/deselected during run setup.
- For MBC-tagged libraries, turn off any adaptor trimming tools included in Illumina's run setup and read processing software applications. Adaptors are trimmed in later processing steps using Agilent software tools to ensure proper processing of the adaptors, including the degenerate molecular barcodes (MBCs) in the adaptor sequences.
- For runs set up using Illumina's LRM, IEM, or BaseSpace applications, refer to Illumina's instructions and support resources for setting up runs with custom library prep kits and index kits in the selected software. For use in these applications, the SureSelect XT HS2 index sequences provided in **Table 87** through **Table 94** should be converted to .tsv/.csv file format or copied to a Sample Sheet according to Illumina's specifications for each application. If you need assistance with SureSelect XT HS2 run setup in your selected application (e.g., generating index files or Sample Sheet templates), contact the SureSelect support team (see **page 2**) or your local representative.

Step 4. Process and analyze the reads

Guidelines are provided below for typical NGS read processing and analysis pipeline steps appropriate for SureSelect XT HS2 DNA libraries. Your NGS analysis pipeline may vary.

• Demultiplex using Illumina's bcl2fastq, BCL Convert or DRAGEN software to generate paired end reads based on the dual indexes and remove sequences with incorrectly paired P5 and P7 indexes. For MBC-tagged libraries, turn off the MBC/UMI trimming options in Illumina's demultiplexing software to allow proper adaptor processing and use of the MBCs by Agilent's NGS software tools.

• The demultiplexed FASTQ data needs to be pre-processed to remove sequencing adaptors and extract the MBC sequences, if present. Both of the Agilent NGS software tools described below include the correct read pre-processing steps for SureSelect XT HS2 DNA libraries.

• Agilent's Alissa Reporter software provides a variety of applications for the complete FASTQ file to variant discovery process for SureSelect-enriched libraries. See **page 109** for more information.

• Agilent's Genomics NextGen Toolkit (AGeNT) software modules provide the tools to process the library read FASTQ files to analysis-ready BAM files for germline or somatic variant analysis workflows. See **page 110** for more information.

• If you have prepared MBC-tagged libraries, but 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 111**.

Using Agilent's Alissa Reporter software for SureSelect XT HS2 DNA NGS 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 germline or somatic SNV, InDel and CNV calls in human samples.

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 variant calling 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:

- Determine the sample co-processing requirements for your application. Sample requirements for germline application CNV analysis are outlined below. Somatic applications directed to tumor/normal analysis require co-processing of the tumor sample and a matched/unmatched reference (normal) sample in the same SureSelect NGS library preparation run. Consult the Alissa Reporter software Help topics from within the software for detailed information on sample requirements for the available applications.

- Alissa Reporter makes germline CNV calls using a co-analysis strategy in which unrelated samples from the same run are used to determine the reference signal for the target sample (no specific reference sample is required for the germline applications). 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 sex 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.
- Alissa Reporter includes applications for germline and somatic analysis of human DNA libraries enriched using a pre-designed or custom SureSelect human probe. Libraries enriched using SureSelect XT HS Human All Exon V7 or V8 probes are analyzed with the corresponding application in Alissa Reporter (*Human All Exon V7 Germline, Human All Exon V8 Germline, Human All Exon V7 Somatic* or *Human All Exon V8 Somatic*). Libraries enriched using other optimized 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 and custom probe designs from SureDesign and setting up a new *Custom* application for each imported design.
- Unmerged and merged FASTQ files are supported. Upload of BAM files or other non-FASTQ file formats is not supported at this time.
- Obtain any required sequence file parameters (e.g., file size or read number limits) from the Alissa Reporter software Help topics available within the software or from the Alissa Reporter Release Notes for the current software version. Key FASTQ file parameters for Alissa Reporter version 1.2 are provided in **Table 77**.

Table 77 FASTQ file parameters for Alissa Reporter software v1.2

Parameter	Value(s)	Notes
Maximum file size	100 GB/file	—
Maximum files uploaded per Alissa Reporter run	768 files	—
Read number allowance per file before subsampling	150M reads for *Human All Exon V7 Germline* or *Human All Exon V8 Germline* application 500M reads for *Custom DNA Germline* application 1.5B reads for *Human All Exon V7 Somatic*, *Human All Exon V8 Somatic,* or *Custom DNA Somatic* application	If the number of reads for a sample is greater than the subsampling limit, reads are randomly subsampled down the limit for the selected application.

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 libraries are MBC-tagged but your sequence analysis pipeline excludes MBCs, you can remove the first 5 bases from Read 1 and Read 2 by masking or trimming before proceeding to downstream analysis.

If demultiplexing using bcl2fastq, MBCs may be masked by including the base mask **N5Y*,I8,I8,N5Y*** (where * is replaced with the remaining read length after subtracting the 5 masked bases, e.g., use **N5Y146,I8,I8,N5Y146** for 2x150 NGS set up as shown in **Table 76** on page 108). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

If demultiplexing using BCL Convert, MBCs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y*;I8;I8;N5Y*** (where * is replaced with read length after trimming, e.g., use **N5Y146;I8;I8;N5Y146** for 2x150 NGS set up as shown in **Table 76** on page 108). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

Alternatively, the first 5 bases may be trimmed from the demultiplexed FASTQ files using a suitable processing tool of your choice, such as seqtk. The AGeNT Trimmer module can also be used to remove the MBCs while trimming adaptor sequences. Non-Agilent adaptor trimmers will fail to remove the MBC sequences from the opposite adaptor (refer to **Figure 24**), which may affect alignment quality.

8 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 78**.

Table 78 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 50**	Qualification of DNA Integrity	Not required	Required
Enzymatic fragmentation duration **page 63**	Duration of the 37°C fragmentation step	15–25 minutes, depending on read length requirements	25 minutes
DNA input for Library Preparation **page 51**	Input amount and means of quantification	10 ng to 200 ng, quantified by Qubit assay	Based on determined DNA integrity (see **Table 19** on page 51 and **Table 20** on page 51)
DNA Shearing **page 53**	Mode of DNA Shearing	2 × 120 seconds	240 seconds (continuous)
Pre-capture PCR **page 64**	Cycle number	8–11	11–14
Sequencing **page 116**	Output augmentation	Per project requirements	1× to 10× based on determined DNA integrity (see **Table 79** and **Table 80** on page 116)

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 79**. 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 79 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 80**. 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 80 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

9 Reference

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

Kit Contents

The SureSelect XT HS2 DNA Kits protocol using the Agilent Bravo NGS Workstation uses the kits listed in **Table 81**. Detailed contents of each of the multi-part component kits listed in **Table 81** are shown in **Table 82** through **Table 85** on the following pages.

Table 81 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 82 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 83 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 84 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 85 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 (see **page 129** through **page 130** for plate maps). Each well contains a single- use aliquot of a specific pair of P7 plus P5 primers.

CAUTION The SureSelect XT HS2 Index Primer Pairs are provided in single-use aliquots. To avoid cross-contamination of libraries, use each well in only one library preparation reaction. Do not retain and re-use any residual volume for subsequent experiments.

The nucleotide sequence of the index portion of each primer is provided in **Table 87** through **Table 93**. 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 86**. 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 strand orientation for your application.

Table 86 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 87 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 88 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 89 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 90 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 91 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 92 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 93 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

Table 94 SureSelect XT HS2 Index Primer Pairs 337–384, provided in red 96-well plate

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

Index Primer Pair Plate Maps

Table 95 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 96 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 97 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 98 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 Protocol using NGS Workstation Option B+ with ODTC protocol.

Enzymatic Fragmentation and Library Preparation

Table 99 Fragmentation master mix - used on page 56

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2 µL	42.5 µL	59.5 µL	76.5 µL	97.8 µL	136.0 µL	253.8 µL
5X SureSelect Fragmentation Buffer (blue cap)	2 µL	42.5 µL	59.5 µL	76.5 µL	97.8 µL	136.0 µL	253.8 µL
SureSelect Fragmentation Enzyme (green cap)	1 µL	21.3 µL	29.8 µL	38.3 µL	48.9µL	68.0 µL	126.9 µL
Total Volume	**5 µL**	**106.3 µL**	**148.8 µL**	**191.3µL**	**244.4 µL**	**340.0 µL**	**634.4 µL**

Table 100 End Repair/dA-Tailing master mix - used on page 57

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
End Repair-A Tailing Buffer (yellow cap or bottle)	16 µL	204 µL	340 µL	476 µL	612 µL	884 µL	1836 µL
End Repair-A Tailing Enzyme Mix (orange cap)	4 µL	51 µL	85 µL	119 µL	153 µL	221 µL	459 µL
Total Volume	**20 µL**	**255 µL**	**425 µL**	**595 µL**	**765 µL**	**1105 µL**	**2295 µL**

Table 101 Ligation master mix - used on page 58

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Ligation Buffer (purple cap or bottle)	23 µL	293.3 µL	488.8 µL	684.3 µL	879.8 µL	1270.8 µL	2737 µL
T4 DNA Ligase (blue cap)	2 µL	25.5 µL	42.5 µL	59.5 µL	76.5 µL	110.5 µL	238 µL
Total Volume	**25 µL**	**318.8 µL**	**531.3 µL**	**743.8 µL**	**956.3 µL**	**1381.3 µL**	**2975 µL**

Table 102 Adaptor Oligo Mix dilution - used on page 58

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2.5 µL	42.5 µL	63.8 µL	85.0 µL	106.3 µL	143.5 µL	276.3 µL
SureSelect XT HS2 Adaptor Oligo Mix (white cap)	5 µL	85.0 µL	127.5 µL	170.0 µL	212.5 µL	287.0 µL	552.5 µL
Total Volume	**7.5 µL**	**127.5 µL**	**191.3 µL**	**255.0 µL**	**318.8 µL**	**430.5 µL**	**828.8 µL**

Table 103 Pre-Capture PCR master mix - used on page 59

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
5× Herculase II Buffer with dNTPs (clear cap)	10 µL	170 µL	255 µL	340µL	425 µL	574 µL	1066 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17 µL	25.5 µL	34 µL	42.5 µL	57.4 µL	106.6 µL
Total Volume	**11 µL**	**187 µL**	**280.5 µL**	**374µL**	**467.5 µL**	**631.4 µL**	**1172.6 µL**

Table 104 Master mix source plate for library preparation runset - used on page 59

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Fragmentation master mix (if using)*	Column 1 (A1-H1)	12.5 µL	17.5 µL	22.5 µL	28.8 µL	40.0 µL	75.0 µL
End Repair-dA Tailing master mix	Column 2 (A2-H2)	31.0 µL	52.0 µL	73.0 µL	94.0 µL	136.0 µL	280.0 µL
Ligation master mix	Column 3 (A3-H3)	36.0 µL	62.0 µL	88.0 µL	114.0 µL	166.0 µL	360.0 µL
Adaptor Oligo Mix dilution	Column 4 (A4-H4)	15.0 µL	22.5 µL	30.0 µL	37.5 µL	52.5 µL	101.3 µL
Pre-Capture PCR Master Mix	Column 5 (A5-H5)	22 µL	33 µL	44 µL	55 µL	77 µL	143 µL

* The fragmentation master mix is only needed for the enzymatic fragmentation workflow. If you are using the mechanical fragmentation workflow, leave column 1 of the master mix source place empty.

Hybridization and Capture

Table 105 Magnetic bead washing mixture - used on page 86

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Streptavidin T1 bead suspension	50 µL	425 µL	825 µL	1225 µL	1.65 mL	2.5 mL	5.0 mL
SureSelect Binding Buffer	0.2 mL	1.7 mL	3.3 mL	4.9 mL	6.6 mL	10 mL	20 mL
Total Volume	**0.25 mL**	**2.125 mL**	**4.125 mL**	**6.125 mL**	**8.25 mL**	**12.5 mL**	**25 mL**

Table 106 Resuspension of magnetic beads - used on page 87

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Binding Buffer	0.2 mL	1.7 mL	3.3 mL	4.9 mL	6.6 mL	10 mL	20 mL

Table 107 Block master mix - used on page 87

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2.5 µL	31.9 µL	53.1 µL	74.4 µL	95.6 µL	138.1 µL	276.3 µL
SureSelect XT HS2 Blocker Mix (blue cap)	5.0 µL	63.8 µL	106.3 µL	148.8 µL	191.3 µL	276.3 µL	552.5 µL
Total Volume	**7.5 µL**	**95.7 µL**	**159.4 µL**	**223.2 µL**	**286.9 µL**	**414.4 µL**	**828.8 µL**

Table 108 Post-Capture PCR master mix - used on page 88

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
5× Herculase II Reaction Buffer with dNTPs (clear cap)	10 µL	170 µL	255 µL	340 µL	425 µL	574 µL	1105 µL
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	17.0 µL	25.5 µL	34.0 µL	42.5 µL	57.4 µL	110.5 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17.0 µL	25.5 µL	34.0 µL	42.5 µL	57.4 µL	110.5 µL
Total Volume	**12.0 µL**	**204.0 µL**	**306.0 µL**	**408.0 µL**	**510.0 µL**	**688.8 µL**	**1326.0 µL**

Table 109 Probe master mix for Probes <3 Mb, 8 rows of wells - used on page 88

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	7.0 µL	89.3 µL	148.8 µL	208.3 µL	267.8 µL	401.6 µL	818.1 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	28.7 µL	58.4 µL
SureSelect Fast Hybridization Buffer (bottle)	6.0 µL	76.5 µL	127.5 µL	178.5 µL	229.5 µL	344.3 µL	701.3 µL
Probe (with design <3.0 Mb)	2.0 µL	25.5 µL	42.5 µL	59.5 µL	76.5 µL	114.8 µL	233.8 µL
Total Volume	**15.5 µL**	**197.6 µL**	**329.4 µL**	**461.1 µL**	**592.9 µL**	**889.4 µL**	**1811.6 µL**

Table 110 Probe master mix for Probes ≥3 Mb, 8 rows of wells - used on page 89

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.0 µL	51.0 µL	85.0 µL	119.0 µL	153.0 µL	229.5 µL	467.5 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	28.7 µL	58.4 µL
SureSelect Fast Hybridization Buffer (bottle)	6	76.5	127.5	178.5	229.5	344.3 µL	701.3
Probe (with design ≥3 Mb)	5.0 µL	63.8 µL	106.3 µL	148.8 µL	191.3 µL	286.9 µL	584.4 µL
Total Volume	**15.5 µL**	**197.6 µL**	**329.4 µL**	**461.1 µL**	**592.9 µL**	**889.4 µL**	**1811.6 µL**

Table 111 Probe master mix for Probes <3 Mb, single row of wells - used on page 89

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	7 µL	10.5 µL	17.5 µL	24.5 µL	31.5 µL	49.0 µL	98.0 µL
RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.3 µL	3.5 µL	7.0 µL
SureSelect Fast Hybridization Buffer (bottle)	6.0 µL	9.0 µL	15.0 µL	21.0 µL	27.0 µL	42.0 µL	84.0 µL
Probe (with design <3.0 Mb)	2.0 µL	3.0 µL	5.0 µL	7.0 µL	9.0 µL	14.0 µL	28.0 µL
Total Volume	**15.5 µL**	**23.3 µL**	**38.8 µL**	**54.3 µL**	**69.8 µL**	**108.5 µL**	**217.0 µL**

Table 112 Probe master mix for Probes ≥3 Mb, single row of wells - used on page 90

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.0 µL	6.0 µL	10.0 µL	14.0 µL	18.0 µL	28.0 µL	56.0 µL
RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.3 µL	3.5 µL	7.0 µL
SureSelect Fast Hybridization Buffer (bottle)	6.0 µL	9.0 µL	15.0 µL	21.0 µL	27.0 µL	42.0 µL	84.0 µL
Probe (with design ≥3 Mb)	5.0 µL	7.5 µL	12.5 µL	17.5 µL	22.5 µL	35.0 µL	70.0 µL
Total Volume	**15.5 µL**	**23.3 µL**	**38.8 µL**	**54.3 µL**	**69.8 µL**	**108.5 µL**	**217.0 µL**

Table 113 Master mix source plate for SSELHyb&Capture_XT_HS2 runset - used on page 90

Master Mix Solution	**Position on Source Plate**	**Volume of master mix added per Well of Eppendorf twin.tec Source Plate**					
		1-Column Runs	**2-Column Runs**	**3-Column Runs**	**4-Column Runs**	**6-Column Runs**	**12-Column Runs**
Block master mix	Column 1 (A1-H1)	11.0 µL	19.0 µL	27.0 µL	34.9 µL	50.9 µL	102.7 µL
Probe Hybridization master mix	Column 2 (A2-H2)	23.3 µL	38.8 µL	54.3 µL	69.8 µL	108.5µL	217.0 µL*
Post-Capture PCR master mix	Column 3 (A3-H3)	23.0 µL	36.0 µL	49.0 µL	62.0 µL	82.0 µL	163.0 µL

* 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.

Quick Reference Tables for Other Reagent Volumes

This section contains tables the 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 114 Genomic DNA Input Volumes

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

Table 115 XT HS2 Index Primer Pairs Volume on Primer Plate

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

Table 116 AMPure XP Bead Volumes for AMPure XP Protocols

Protocol or Runset	Volume of AMPure Beads per Well*
EnzymaticFrag_LibPrep_XT_HS2 and MechanicalFrag_LibPrep_XT_HS2	80 µL (library preparation) 50 µL (pre-capture PCR)
AMPureXP_XT_HS2_ILM (Case: Concentration of Pool)	180 µL
SSELHyb&Capture_XT_HS2	50 µL (post-capture PCR)

* 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 117 Water and Ethanol Volumes for Reservoirs

Reagent	Volume per Reservoir
70% ethanol in Agilent deep well reservoir, with lid	Library prep runsets: 100 mL All other protocols/runsets: 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 over-amplified. 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 69**. 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 88**, 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 purification with the AMPure XP beads depends upon using the correct ratio of sample to 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.

If regions with high GC content are under-represented in library sequencing results

- ✔ High GC-dropout may indicate that an extended hybridization time is needed to improve capture.
  - For libraries target-enriched without the Hyb Extension option described in **Table 66** on page 93, repeat the SSELHyb&Capture_XT_HS2 runset with the Hyb Extension check box marked.

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 Hyb Extension option described in **Table 66** on page 93, repeat the SSELHyb&Capture_XT_HS2 runset without the Hyb Extension option.

If an automated runset appears to freeze/hang in VWorks before completion

✔ At times, the VWorks software and NGS Workstation can freeze while a runset is running. If you see that an in-progress runset is no longer progressing, follow the steps below.

- On your PC, close all applications other than VWorks.
- In VWorks, if you are in full screen view, click the **Full Screen** toggle button to return to the tabbed view.
- On the Main Log tab, check if any of the devices encountered an error.
  - If there is an error in the main log, check the description and make the needed corrections based on that information.
  - If the main log does not display an error, then the freeze likely occurred during initialization of one of the protocols in the runset. Continue the steps below to restart the runset from the point it left off.
- Under **Controls**, click **Pause**. The **Schedule Paused** dialog box opens. (If the Pause button is unresponsive, see the following check mark below.)
- Click **Abort process**. The **Abort process** dialog box opens.
- Select **Abort all the protocols in the runset** and click **OK**.
- Click the Runset Manager tab. This tab lists the protocols contained in the current runset. Each row is a separate protocol.
- Select the rows for the protocols in the runset that already completed before the freeze. Press the Shift key to select a range of rows. With the rows for the completed protocols selected, click **Delete run**. The top row in the Runset Manager is now the protocol at which the runset froze.
- Right-click on the top row of the Runset Manager. In the menu that opens, click **Adjust run start time and dependencies**. A dialog box opens.
- In the dialog box, under **Determine when the protocol will start**, select **As soon as possible** and click **Finish**. The runset will resume with the protocol at which the freeze occurred.

✔ If the VWorks software and NGS Workstation freeze while a runset is running and the Pause button is unresponsive, follow the steps below.

- Make note of the runset progress in VWorks, specifically which protocol modules were completed before the freeze and where the runset left off.
- Force VWorks to close.
  - Press Ctrl+Alt+Delete and click **Task Manager**.
  - In the Task Manager window, select the VWorks software and click **End task**.
- Relaunch the SureSelect XT HS2 DNA form in VWorks using the instructions in **"Opening the SureSelect XT HS2 DNA form"** on page 34.
- Set up the Run Parameters on the screen as you had them configured when the system froze.
- Click **Run Selected Runset**.

- When you see the notification message “This runset contains protocols that will start running as soon as possible”, click **Cancel** in the message.
- If you are in full screen view, click the **Full Screen** toggle button to return to the tabbed view.
- Click the Runset Manager tab. This tab lists the protocols contained in the selected runset. Each row is a separate protocol.
- Select the rows for the protocols in the runset that already completed before the freeze. Press the Shift key to select a range of rows. With the rows for the completed protocols selected, click **Delete run**. The top row in the Runset Manager is now the protocol at which the runset froze.
- Right-click on the top row of the Runset Manager. In the menu that opens, click **Adjust run start time and dependencies**. A dialog box opens.
- In the dialog box, under **Determine when the protocol will start**, select **As soon as possible** and click **Finish**. The runset will resume with the protocol at which the freeze occurred.

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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 Bravo NGS Workstation Option B+.

Version A0, August 2023

G9985-90015

Agilent