SureSelect XT HS2 DNA Kits for Library Prep (+/– MBC) and Target Enrichment (Fast/Overnight Hyb) with Optional Pre-Capture Pooling

Automated using Agilent Bravo NGS Workstation (Option B)

For Illumina Platform NGS

Protocol

Version A2, October 2023

SureSelect platform manufactured with Agilent SurePrint technology.

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

This protocol applies only to version B1.1.2 of the SureSelect XT HS2 DNA VWorks Form for Option B (XT_HS2_ILM_v.B1.1.2.VWForm).

Notices

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Agilent Technologies, Inc.
5301 Stevens Creek Blvd
Santa Clara, CA 95051 USA

Acknowledgment

Technical Support

For US and Canada

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

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In this Guide...

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

1 Before You Begin

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

2 Using the Agilent Bravo NGS Workstation (Option B) for SureSelect Target Enrichment

This chapter contains an orientation to the NGS Workstation, an overview of the SureSelect XT HS2 target enrichment workflow, and considerations for designing SureSelect experiments for automated processing using the NGS Workstation.

3 Preparation of AMPure XP Bead Plates

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

4 Sample Preparation using +/- MBC Adaptors

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

5 Hybridization (Fast)

This chapter describes the steps to hybridize the DNA library or library pool to the Probe using the fast hybridization method. The DNA libraries are hybridized to a SureSelect or ClearSeq Probe using single-plex or multi-plex hybridization in a thermal cycler program in which the hybridization segment is 1 to 2 hours.

6 Hybridization (Overnight)

This chapter describes the steps to hybridize the DNA library or library pool to the Probe using the overnight hybridization method. The DNA libraries are hybridized to a SureSelect or ClearSeq Probe using single-plex or multi-plex hybridization in a thermal cycler program in which the hybridization segment is 16 to 24 hours.

7 Capture and Amplification

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

8 NGS and Analysis Guidelines

This chapter provides sample pooling instructions for the post-capture pooling workflow as well as instructions and resources for the sequencing and analysis steps.

9 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.

10 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 G9985-90011.

NOTE

This Protocol describes automated DNA sample processing for SureSelect XT HS2 Target Enrichment using version B1.1.2 of the SureSelect XT HS2 DNA VWorks Form for the NGS Workstation.

For automated sample processing procedures using a previous version of the SureSelect XT HS2 DNA VWorks Form for the NGS Workstation, see the following Agilent publication G9985-90010.

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

G9983-90000 – for post-capture pooling workflow with fast hybridization
G9985-90000 – for pre-capture pooling workflow with fast hybridization
G9956-90000 – for post-capture pooling workflow with overnight hybridization
G9957-90000 – for pre-capture pooling workflow with overnight hybridization

NOTE

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

Procedural Notes

- Certain protocol steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the NGS Workstation to allow rapid and efficient plate transfer.
- If your thermal cycler is compatible with the use of compression pads, add a compression pad whenever you load a plate that was sealed with the PlateLoc thermal microplate sealer. The pad improves contact between the plate and the heated lid of the thermal cycler.
- Prepare and load the NGS Workstation as detailed in each of the protocol steps before initiating each automated protocol run. When loading plates in the Labware MiniHub of the NGS Workstation, always place plates in the orientation shown in **Figure 4** on page 53.
- 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.

SureSelect XT HS2 DNA Kits and Supported Workflows

SureSelect XT HS2 DNA kits are available in a variety of kit configurations supporting several workflow options.This protocol provides instructions for using the NGS Workstation to automate workflows that include SureSelect XT HS2 DNA library preparation (+/- duplex molecular barcodes or MBCs) and target enrichment (with either fast or overnight hybridization) with the option to perform pre-capture library pooling.

To determine the materials needed for your research design, first select the appropriate SureSelect XT HS2 DNA Kits for the workflow from either **Table 1** (for pre-capture pooling workflows) or **Table 2** (for post-capture pooling workflows). Unless otherwise noted, you must purchase both a Library Preparation Kit (left column) and a Target Enrichment Kit (right column). Select the appropriate kits based on pooling method, MBC usage, and hybridization method.

See **"Automation Protocols used in the Workflow"** on page 30 for descriptions and usage information for all supported workflow modulations. See **"Kit Contents"** on page 168 for a list of the contents of each SureSelect XT HS2 Kit.

Table 1 SureSelect XT HS2 DNA Kits for Pre-Capture Pooling

SureSelect XT HS2 DNA Library Preparation Kit	SureSelect XT HS2 DNA Target Enrichment Kit*
For Pre-Capture Pooling with MBC-tagged Libraries	
SureSelect XT HS2 DNA Library Preparation Kit, 96 reactions: • Agilent p/n G9985A (with Index Pairs 1–96) • Agilent p/n G9985B (with Index Pairs 97–192) • Agilent p/n G9985C (with Index Pairs 193–288) • Agilent p/n G9985D (with Index Pairs 289–384)	Fast hybridization: • Agilent p/n G9987A, SureSelect XT HS2 DNA Target Enrichment Kit, 12 Hybridizations **-- OR --** Overnight hybridization: • Agilent p/n G9957A†, SureSelect XT HS2 Target Enrichment Kit, Overnight Hybridization, 12 Hybridizations
For Pre-Capture Pooling with MBC-free Libraries	
SureSelect XT HS2 DNA Library Preparation Kit with MBC-free adaptors, 96 reactions: • Agilent p/n G9956A (with Index Pairs 1–96) • Agilent p/n G9956B (with Index Pairs 97–192) • Agilent p/n G9956C (with Index Pairs 193–288) • Agilent p/n G9956D (with Index Pairs 289–384)	Fast hybridization: • Agilent p/n G9987A, SureSelect XT HS2 DNA Target Enrichment Kit, 12 Hybridizations **-- OR --** Overnight hybridization: • Agilent p/n G9957A†, SureSelect XT HS2 Target Enrichment Kit, Overnight Hybridization, 12 Hybridizations

* The Target Enrichment Kits provide 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 2 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 8 Target Enrichment Kits. Pre-capture pooling of samples can use the configuration of either 8 samples/pool or 16 samples/pool.

† Agilent p/n G9957A includes Streptavidin Beads. Separate purchase of Streptavidin Beads is not necessary.

Table 2 SureSelect XT HS2 Kits for Post-Capture Pooling

SureSelect XT HS2 DNA Library Preparation Kit	SureSelect XT HS2 DNA Target Enrichment Kit
Post-Capture Pooling with MBC-tagged Libraries *with Fast Hybridization*	
SureSelect XT HS2 DNA Reagent Kit, 96 reactions • Agilent p/n G9983A (with Index Pairs 1–96) • Agilent p/n G9983B (with Index Pairs 97–192) • Agilent p/n G9983C (with Index Pairs 193–288) • Agilent p/n G9983D (with Index Pairs 289–384) **-- OR --** SureSelect XT HS2 DNA Reagent Kit with AMPure XP and Streptavidin Beads, 96 reactions* • Agilent p/n G9984A (with Index Pairs 1–96) • Agilent p/n G9984B (with Index Pairs 97–192) • Agilent p/n G9984C (with Index Pairs 193–288) • Agilent p/n G9984D (with Index Pairs 289–384)	N/A – *The SureSelect XT HS2 DNA Reagent Kits listed on the left contain reagents for both library preparation and target enrichment*
Post-Capture Pooling with MBC-tagged Libraries *with Overnight Hybridization*	
SureSelect XT HS2 DNA Library Preparation Kit, 96 reactions • Agilent p/n G9985A (with Index Pairs 1–96) • Agilent p/n G9985B (with Index Pairs 97–192) • Agilent p/n G9985C (with Index Pairs 193–288) • Agilent p/n G9985D (with Index Pairs 289–384)	Agilent p/n G9957B†, SureSelect XT HS2 Target Enrichment Kit, Overnight Hybridization, 96 Hybridizations
Post-Capture Pooling with MBC-free Libraries	
SureSelect XT HS2 Library Preparation Kit with MBC-free adaptors, 96 reactions • Agilent p/n G9956A (with Index Pairs 1–96) • Agilent p/n G9956B (with Index Pairs 97–192) • Agilent p/n G9956C (with Index Pairs 193–288) • Agilent p/n G9956D (with Index Pairs 289–384)	Fast Hybridization • Agilent p/n G9987B†, SureSelect XT HS2 Target Enrichment Kit, 96 Hybridizations Overnight Hybridization • Agilent p/n G9957B†, SureSelect XT HS2 Target Enrichment Kit, Overnight Hybridization, 96 Hybridizations

* These kits include AMPure XP Beads and Streptavidin Beads. Separate purchase of these reagents is not necessary.

† Agilent p/n G9957B and G9987B include Streptavidin Beads. Separate purchase of Streptavidin Beads is not necessary.

Additional Materials Required

The additional materials required to complete the SureSelect XT HS2 DNA automation protocol will vary based on the following considerations:

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

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

- See **"Required Reagents and Equipment for All Sample and Workflow Types"** on page 13 for the reagents and equipment required for all sample types and workflows.
- See **"Compatible Probes"** on page 16 for compatible probes.
- See **"Additional Materials Based on Workflow Modulations and Options"** on page 18 for additional materials you may need based on the specifics of your workflow.

Required Reagents and Equipment for All Sample and Workflow Types

Table 3 and **Table 4** list the reagents and equipment that are required to complete this protocol regardless of the sample type or selected workflow modulations. **Table 5** lists the options for nucleic acid analysis platforms.

Table 3 Required Reagents--All Sample and Workflow Types

Description	Vendor and part number
AMPure XP Kit* 60 mL 450 mL	Beckman Coulter Genomics p/n A63881 p/n A63882
Dynabeads MyOne Streptavidin T1* 10 mL 50 mL	Thermo Fisher Scientific p/n 65602 p/n 65604D
1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276
Qubit BR dsDNA Assay Kit 100 assays 500 assays	Thermo Fisher Scientific p/n Q32850 p/n Q32853
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

* Separate purchase **not** required when using a SureSelect XT HS2 DNA Kit that includes this reagent. These exceptions are noted in **Table 1** and **Table 2**.

Table 4 Required Equipment--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 G5522A (VWorks software version 13.1.0.1366 OR Agilent p/n G5574AA (VWorks software version 13.1.0.1366)
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Thermal cycler and accessories (including compression pads, if compatible)	Various suppliers *Important*: Not all PCR plate types are supported for use in the VWorks automation protocols for the NGS Workstation. Select a thermal cycler that is compatible with one of the supported PCR plate types. See supported plate types in the listing below.
PCR plates compatible with the NGS Workstation and associated VWorks automation protocols	Only the following PCR plates are supported: • 96 ABI PCR half-skirted plates (MicroAmp Optical plates), Thermo Fisher Scientific p/n N8010560 • 96 Agilent semi-skirted PCR plate, Agilent p/n 401334 • 96 Eppendorf twin.tec half-skirted PCR plates, Eppendorf p/n 951020303 • 96 Eppendorf twin.tec PCR plates (full-skirted), Eppendorf p/n 951020401
Eppendorf twin.tec full-skirted 96-well PCR plates	Eppendorf p/n 951020401 or 951020619
Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 19 mm height – used when NGS Workstation setup calls for **Agilent Shallow Well Reservoir**	Agilent p/n 201254-100
Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 44 mm height – used when NGS Workstation setup calls for **Agilent Deep Well Reservoir**	Agilent p/n 201244-100
Agilent Storage/Reaction Microplates, 96 wells, 1 mL/round well – used when NGS Workstation setup calls for **Agilent Deep Well Plate** or **Agilent DW Plate**	Agilent p/n 203426-100
Agilent Storage/Reaction Microplates, 96 wells, 2 mL/square well – used when NGS Workstation setup calls for **Waste Plate (Agilent 2 mL Square Well)**	Agilent p/n 201240-100
Nucleic acid surface decontamination wipes	DNA Away Surface Decontaminant Wipes, Thermo Scientific p/n 7008, or equivalent
Qubit Fluorometer	Thermo Fisher Scientific p/n Q33238
Qubit Assay Tubes	Thermo Fisher Scientific p/n Q32856
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Microcentrifuge	Eppendorf microcentrifuge, model 5417C or equivalent
Plate centrifuge	Labnet International MPS1000 Mini Plate Spinner, p/n C1000 or equivalent
Magnetic separator	DynaMag-50 magnet, Thermo Fisher Scientific p/n 123-02D or equivalent
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

Table 4 Required Equipment--All Sample and Workflow Types (continued)

Description	Vendor and Part Number
Sterile, nuclease-free aerosol barrier pipette tips	general laboratory supplier
Vacuum concentrator – used to concentrate samples (if needed) in the overnight hybridization, post-capture pooling workflow	Savant SpeedVac, model DNA120, or equivalent
Vortex mixer	general laboratory supplier
Ice bucket	general laboratory supplier
Powder-free gloves	general laboratory supplier

Table 5 Nucleic Acid Analysis Platform Options -- Select One

Description	Vendor and part number
Agilent 4200/4150 TapeStation	Agilent p/n G2991AA/G2992AA
Consumables:	
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
RNA ScreenTape	Agilent p/n 5067-5576
RNA ScreenTape Sample Buffer	Agilent p/n 5067-5577
RNA ScreenTape Ladder	Agilent p/n 5067-5578
High Sensitivity RNA ScreenTape	Agilent p/n 5067-5579
High Sensitivity RNA ScreenTape Sample Buffer	Agilent p/n 5067-5580
High Sensitivity RNA ScreenTape Ladder	Agilent p/n 5067-5581
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
Consumables:	
RNA 6000 Pico Kit	Agilent p/n 5067-1513
RNA 6000 Nano Kit	Agilent p/n 5067-1511
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
Agilent 5200/5300/5400 Fragment Analyzer Instrument	Agilent p/n M5310AA/M5311AA/M5312AA
Consumables:	
RNA Kit (15NT)	p/n DNF-471-0500
HS RNA Kit (15NT)	p/n DNF-472-0500
NGS Fragment Kit (1-6000 bp)	p/n DNF-473-0500
HS NGS Fragment Kit (1-6000 bp)	p/n DNF-474-0500

Compatible Probes

Table 6 lists the probes compatible with fast hybridization. **Table 7** lists the probes compatible with overnight hybridization. The tables distinguish between probes suitable for workflows with pre-capture pooling of DNA libraries and probes suitable for workflows with post-capture pooling of DNA libraries.

Table 6 Compatible Probes for Fast Hybridization

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 PreCap Clinical Research Exome V4 (12 Hybs)	S34226363	Agilent p/n 5280-0030
	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 5280-0021
	SSel XT HS and XT Low Input Human All Exon V7 (96 Hybs)	S31285117	Agilent p/n 5191-4029

Table 7 Compatible Probes for Overnight Hybridization

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 PreCap Clinical Research Exome V4 (12 Hybs)	S34226363	Agilent p/n 5280-0030
	SureSelect Pre-Capture Pooling Human All Exon V7 (12 Hybs)	S31285117	Agilent p/n 5191-5735
	SureSelect XT2 Clinical Research Exome V2 (12 Hybs)	S30409818	Agilent p/n 5190-9501
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 5280-0021
	SSel XT HS and XT Low Input Human All Exon V7, 96 Hybs	S31285117	Agilent p/n 5191-4029
	SureSelect XT Clinical Research Exome V2, 96 Hybs	S30409818	Agilent p/n 5190-9492

NOTE

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

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

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

Additional Materials Based on Workflow Modulations and Options

Table 8 lists additional required materials you may need depending on the DNA sample type and your selected DNA fragmentation method.

Table 9 lists optional materials.

Table 8 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 9 Supplier Information for Optional Materials

Description	Vendor and part number
High-quality gDNA purification system, for example: QIAamp DNA Mini Kit 50 Samples 250 Samples	 Qiagen p/n 51304 p/n 51306
Tween 20 *– Required if storing the libraries at –20°C prior to sequencing.*	Sigma-Aldrich p/n P9416-50ML

2 Using the Agilent Bravo NGS Workstation (Option B) for SureSelect Target Enrichment

This chapter contains an orientation to the NGS Workstation, an overview of the SureSelect XT HS2 target enrichment workflow, and considerations for designing SureSelect experiments for automated processing using the NGS Workstation.

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 10**.

Review the user guides listed in **Table 10** (available at www.agilent.com) to become familiar with the general features and operation of the NGS Workstation components. Instructions for using the Bravo platform and other NGS Workstation components specifically for the SureSelect XT HS2 Target Enrichment workflow are detailed in this user guide.

Table 10 NGS Workstation components User Guide reference information

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

About the Bravo Platform

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

Bravo Platform Deck

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

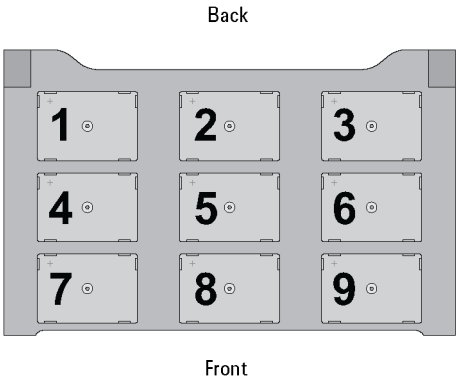


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

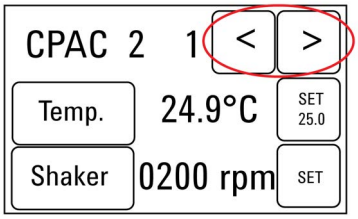
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

Bravo deck heat block temperatures may be changed using the Inheco Multi TEC Control device touchscreen as described in the steps below. See **Table 11** for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

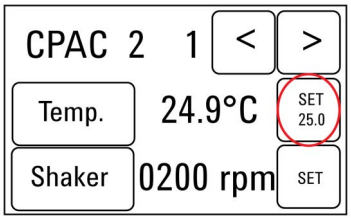
Table 11 Inheco Multi TEC Control touchscreen designations

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

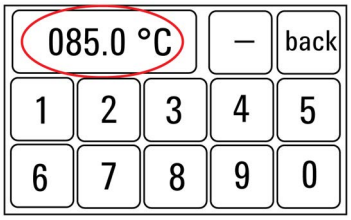
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



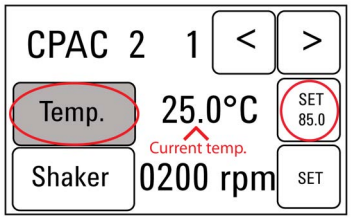
2 To set the temperature of the selected block, press the SET button.



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



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



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

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

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

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

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

3 Press the START button.

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

VWorks Automation Control Software

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

NOTE

The instructions in this manual are compatible with VWorks software version 13.1.0.1366 and version B1.1.2 of the SureSelect XT HS2 DNA VWorks Form.

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

Logging in to the VWorks software

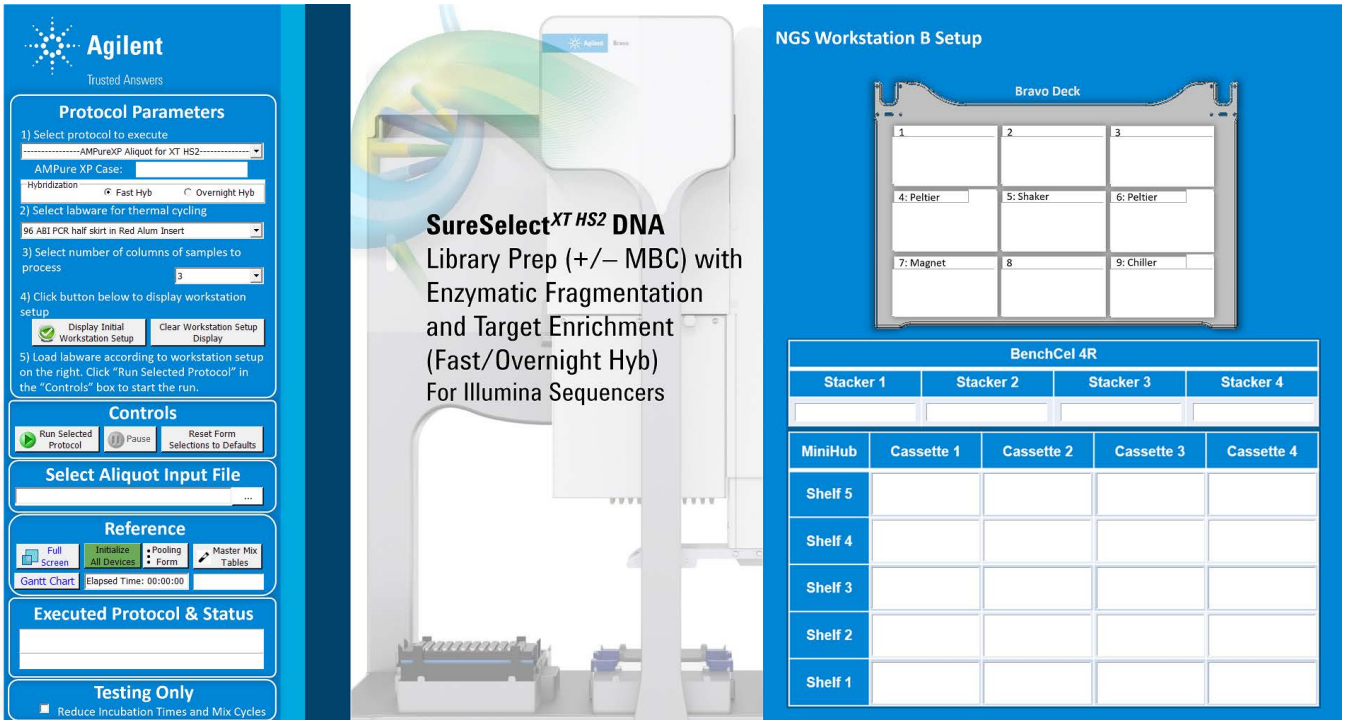
1. Double-click the VWorks icon or the XT HS2 VWorks Form shortcut on the Windows desktop to start the VWorks software.
2. If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
3. In the User Authentication dialog, type your VWorks user name and password, and click **OK**. (If no user account is set up, contact the administrator.)

VWorks protocol and runset files

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

Using the SureSelect XT HS2 DNA Form to setup and start a run

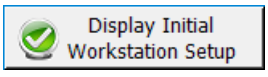
Use the SureSelect XT HS2 DNA VWorks form, shown below, to set up and start each SureSelect automation protocol or runset.



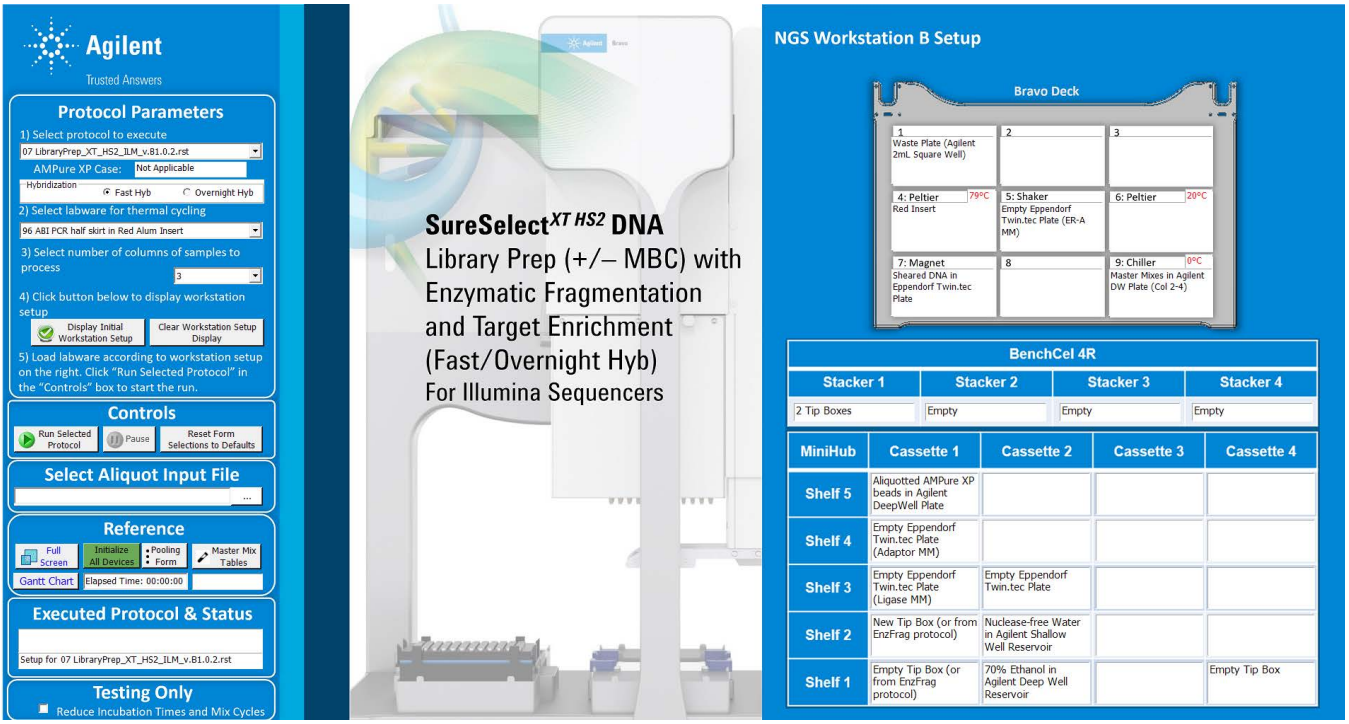
1 Open the form using the shortcut (shown below) on your desktop, or by opening the file **XT_HS2_ILM_v.B1.1.2.VWForm** in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Forms**.

2 Use the form drop-down menus to select the appropriate SureSelect workflow step and number of columns of samples for the run.

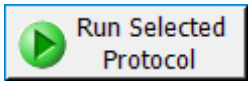
3 Once all run parameters have been specified on the form, click **Display Initial Workstation Setup**.



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



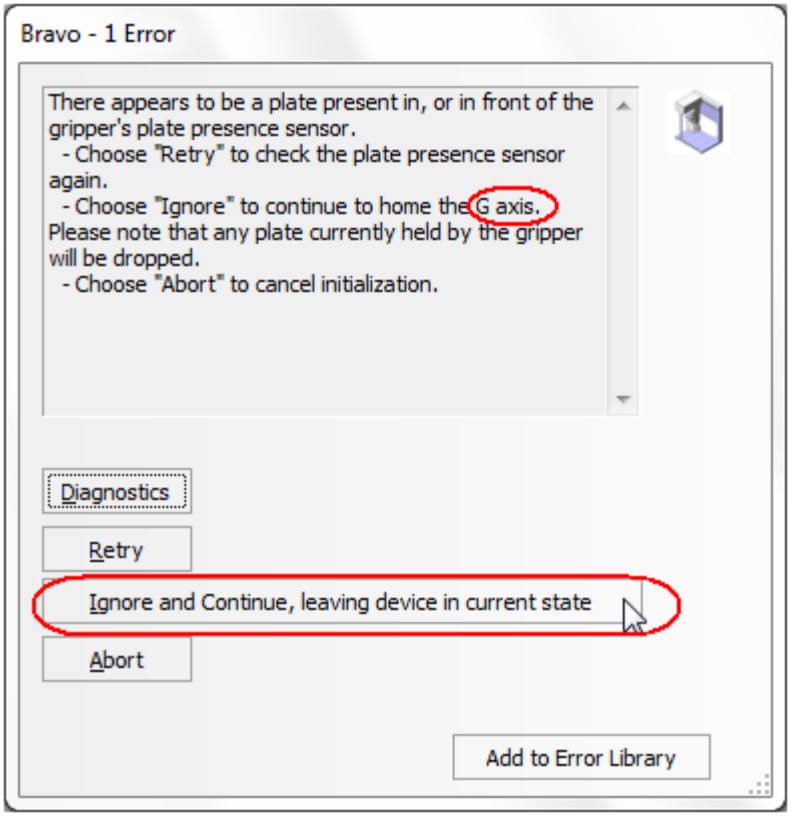
4 After verifying that the NGS Workstation has been set up correctly, click **Run Selected Protocol**.



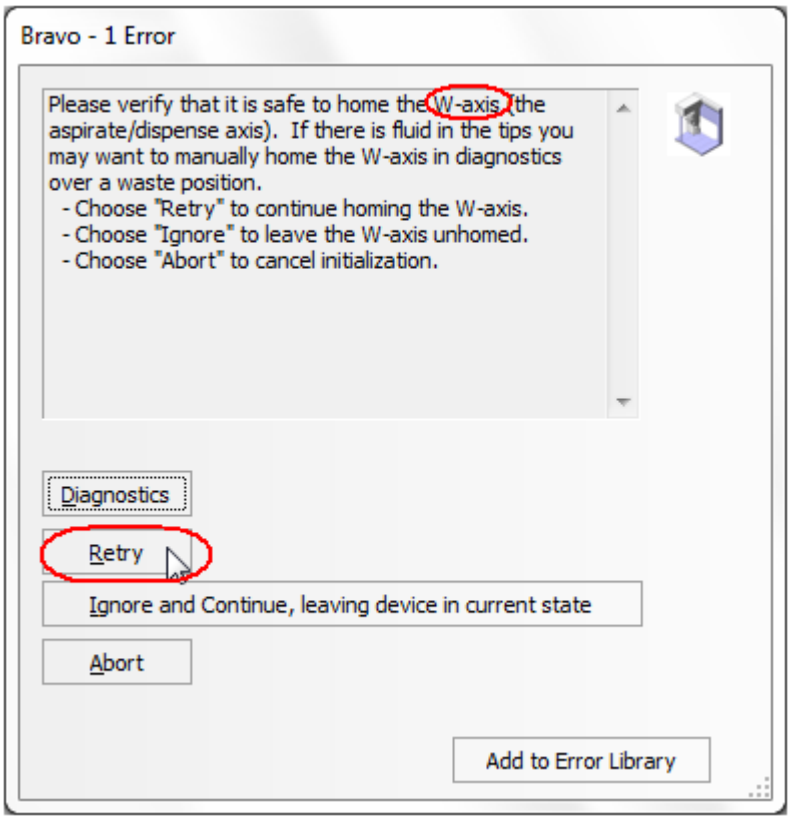
Error messages encountered at start of run

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.



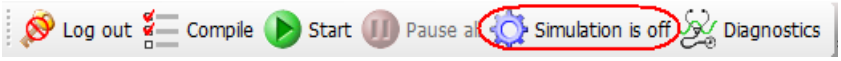
2 If you encounter the W-axis error message shown below, select **Retry**.



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.

Overview of the SureSelect Target Enrichment Procedure

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

Each SureSelect XT HS2 DNA Library Preparation Kit or Reagents Kit offers four different plates of index pairs to allow for multiplexed sequencing (refer to **"Index Primer Pair Plate Maps"** on page 182). 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** for a summary of the VWorks protocols used during the workflow. Then, see **Preparation of AMPure XP Bead Plates**, **Sample Preparation using +/- MBC Adaptors**, **Hybridization (Fast)**, **Hybridization (Overnight)**, and **Capture and Amplification** chapters for complete instructions for use of the VWorks protocols for sample processing.

The SureSelect XT HS2 DNA library preparation protocol is compatible with both high-quality gDNA prepared from fresh or fresh frozen samples and lower-quality DNA prepared from FFPE samples, using a DNA input range of 10 to 200 ng DNA.

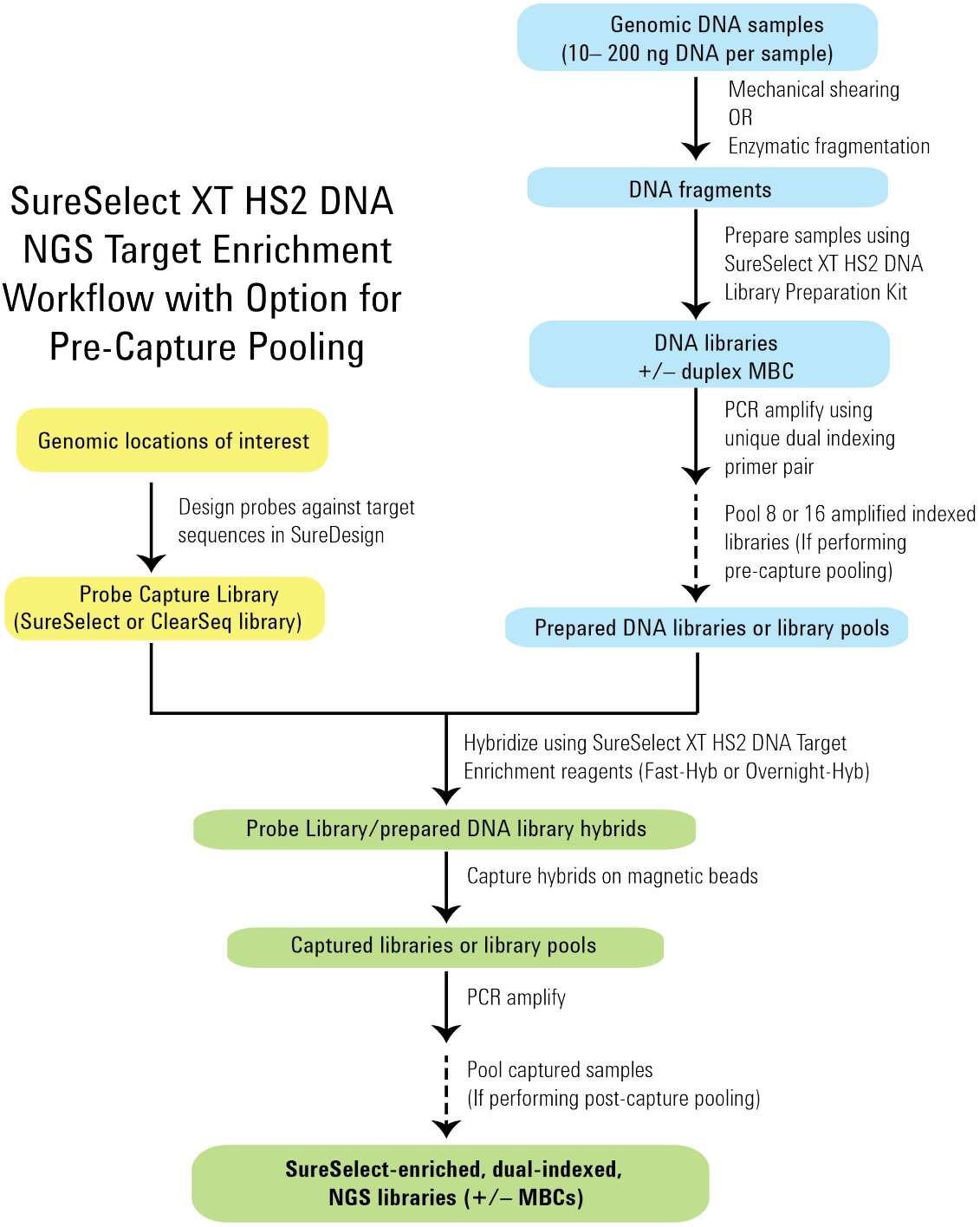


Figure 2 Overall sequencing sample preparation workflow

Automation Protocols used in the Workflow

Table 12 Overview of VWorks protocols and runsets

Workflow Step	Substep	VWorks Protocols Used for NGS Workstation automation
AMPure XP Bead Aliquoting	Aliquot AMPure XP beads for use in the Library Prep runset	AMPureXP_Aliquot (Case Library Prep)
	Aliquot AMPure XP beads for use in the Pre-Capture PCR purification protocol	AMPureXP_Aliquot (Case Pre-Capture PCR)
	Aliquot AMPure XP Beads for use in the Pre-Capture Pooling protocol for concentrating the DNA	AMPureXP_Aliquot (Case Concentration of Pool)
	Aliquot AMPure XP Beads for use in the Post-Capture PCR purification protocol	AMPureXP_Aliquot (Case Post-Capture PCR)
Enzymatic DNA Fragmentation*	Shear DNA samples using enzymatic fragmentation	05 EnzFrag_XT_HS2_ILM
	Dilute fragmented samples to appropriate concentration	06 EnzFrag_Dil_XT_HS2_ILM
Library Preparation	Prepare duplex DNA libraries, with or without molecular barcodes	Runset 07 LibraryPrep_XT_HS2_ILM
	Amplify indexed DNA libraries with unique dual indexing primer pair	08 Pre-CapPCR_XT_HS2_ILM
	Purify indexed DNA libraries using AMPure XP beads in preparation for multi-plex hybridization	AMPureXP_XT_HS2_ILM (Case Pre-Cap PCR - MultiPlex)
	Purify indexed DNA libraries using AMPure XP beads in preparation for single-plex hybridization	AMPureXP_XT_HS2_ILM (Case Pre-Cap PCR - SinglePlex)
	Analyze indexed DNA libraries using Agilent TapeStation platform	10 TS_D1000
Library Pooling (for **pre-capture pooling** workflow)	Pool indexed DNA libraries in pools of 8 or 16	PreCapture_Pooling *This protocol is set up and executed from the XT HS2 Pooling VWorks Form*
Multi-Plex Pre-Hybridization (for **pre-capture pooling** workflow)	Dilute pooled samples of indexed DNA libraries to normalize volumes to 100 µL	12b Aliquot_Water
	Concentrate pooled samples to 24 µL for hybridization	AMPureXP_XT_HS2_ILM (Case Concentration of Pool)
Single-Plex Pre-Hybridization (for **post-capture pooling** workflow)	Aliquot 500-1000 ng (for fast hybridization) or 500-2000 ng (for overnight hybridization) of prepped libraries	12a Aliquot_Libraries
Hybridization and Capture	Hybridize prepped libraries or library pools (target enrichment)	13 Hyb_XT_HS2_ILM
	Capture and wash DNA hybrids	Runset 14 SSELCapture&Wash_XT_HS2

Table 12 Overview of VWorks protocols and runsets (continued)

Workflow Step	Substep	VWorks Protocols Used for NGS Workstation automation
Post-Capture Sample Processing	Amplify target-enriched libraries or library pools	15 Post-CapPCR_XT_HS2_ILM
	Purify enriched, amplified libraries or library pools using AMPure XP beads	AMPureXP_XT_HS2_ILM (Case Post-Capture PCR)
	Analyze final libraries or library pools using Agilent TapeStation platform	17 TS_HighSensitivity_D1000
	For post-capture pooling workflow, pool indexed DNA libraries	18 Aliquot_Captures

* To shear DNA samples mechanically, rather than enzymatically, perform liquid handing steps manually on the Covaris Sample Preparation System (see **"Method 2. Prepare fragmented DNA by mechanical shearing"** on page 57). The XT HS2 VWorks Form does not include an automation protocol for mechanical shearing.

Workflow Modulations

The SureSelect XT HS2 DNA target enrichment workflow can be modulated for different applications as described below and summarized in **Table 13** on page 33.

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

DNA Fragmentation Method Automated Enzymatic Fragmentation protocols are provided to support enzymatic fragmentation with automated liquid handling steps (see **"Method 1: Prepare fragmented DNA by enzymatic fragmentation"** on page 50). Alternatively, DNA can be mechanically sheared using manual liquid handling steps without automation (see **"Method 2. Prepare fragmented DNA by mechanical shearing"** on page 57).

MBC Usage The automated SureSelect XT HS2 DNA workflow supports library preparation with either MBC-tagged adaptors or MBC-free adaptors. Agilent offers SureSelect XT HS2 DNA kits for both types of adaptors in the adaptor oligo mix.

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 with the Probe and subsequent PCR amplification of the captured libraries, pool multiple indexed libraries together prior to sequencing. The allowable number of libraries per pool is dependent on the output specifications of the sequencing platform and the amount of sequencing data required.

Hybridization Time The automated SureSelect XT HS2 DNA workflow can prepare hybridization reactions for either an overnight hybridization or a fast hybridization. These methods use different SureSelect XT HS2 DNA reagents and require a different setup on the VWorks form.

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

Table 13 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 164 for summary of protocol modifications.
DNA Fragmentation Method	Enzymatic Fragmentation	Use the Enzymatic Fragmentation automation protocols to perform enzymatic fragmentation of DNA samples. The protocol EnzFrag_XT_HS2_ILM executes the liquid handling steps for the enzymatic fragmentation reactions. The protocol EnzFrag_Dil_XT_HS2_ILM directs the dilution of the fragmented samples to the concentration needed for library preparation. Requires purchase of SureSelect Enzymatic Fragmentation Kit, 96 Reactions Automation (Agilent p/n 5191-6764)
	Mechanical (Covaris) Shearing	Perform mechanical shearing on Covaris Sample Preparation System using manual liquid handling (no automated protocol). Requires purchase of Covaris Sample Preparation System and consumables (see **"Additional Required Materials based on DNA Sample Type/Fragmentation Method"** on page 18).
MBC Usage	MBC-tagged Adaptors	For library preparation, use a SureSelect XT HS2 DNA Kit that contains MCB-tagged adaptors in the adaptor oligo mix. Refer to **Table 1** on page 11 and **Table 2** on page 12.
	MBC-free Adaptors	For library preparation, use a SureSelect XT HS2 DNA Kit that contains MCB-free adaptors in the adaptor oligo mix. Refer to **Table 1** on page 11 and **Table 2** on page 12.
Pooling Strategy	Pre-Capture Pooling	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 1** on page 11.
	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 2** on page 12.
Hybridization Time	Fast	For target enrichment, use a SureSelect XT HS2 DNA Kit that is compatible with fast hybridization. Refer to **Table 1** on page 11 and **Table 2** on page 12. In the VWorks form, make sure that the **Fast hyb** option is selected when running the AMPureXP_XT_HS2_ILM (Pre-Cap PCR – SinglePlex or MultiPlex) protocol, Aliquot_Libraries protocol, AMPureXP_XT_HS2_ILM protocol, and Hyb_XT_HS2_ILM protocol.
	Overnight	For target enrichment, use a SureSelect XT HS2 DNA Kit that is compatible with overnight hybridization. Refer to **Table 1** on page 11 and **Table 2** on page 12. In the VWorks form, make sure that the **Overnight** option is selected when running the AMPureXP_XT_HS2_ILM (Pre-Cap PCR – SinglePlex or MultiPlex) protocol, Aliquot_Libraries protocol, AMPureXP_XT_HS2_ILM protocol, and Hyb_XT_HS2_ILM protocol.

Experimental Setup Considerations for Automated Runs

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

Table 14 Columns to Samples Equivalency

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

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

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

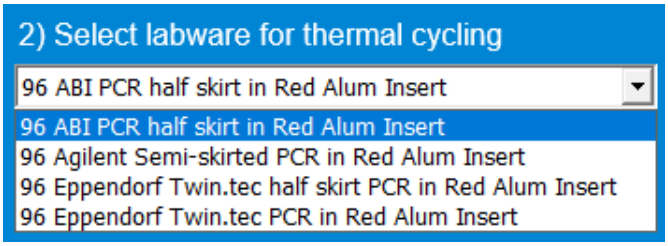
- The NGS Workstation processes samples column-wise beginning at column 1. The gDNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- At the hybridization step (see **Figure 2**), you can add a different Probe to each row of the plate. Plan your experiment such that each prepared DNA library corresponds to the appropriate probe row in the sample plate.
- For post-capture amplification (see **Figure 2**), different probes can require different amplification cycle numbers, based on the probe design sizes. It is most efficient to process similar-sized probes on the same plate. See **Table 103** on page 136 to determine which probes may be amplified on the same plate.

Considerations for Equipment Setup

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

PCR Plate Type Considerations

Automation protocols include several liquid-handling steps in which reagents are dispensed to PCR plates in preparation for transfer to a thermal cycler. For these steps, you must specify on the VWorks Form which PCR plate type will be used to allow correct configuration of the liquid handling components. Before you begin the automation protocol, make sure that you are using a supported PCR plate type. The PCR plate type to be used in the protocol is specified using the menu below. Vendor and part number information is provided for the supported plate types in **Table 15**.



CAUTION

The plates listed in **Table 15** are compatible with the NGS Workstation and associated VWorks automation protocols, designed to support use of various thermal cyclers.

Do not use PCR plates that are not listed in **Table 15**, even if they are compatible with your chosen thermal cycler.

Table 15 Ordering information for supported PCR plates

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

3 Preparation of AMPure XP Bead Plates

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

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

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

The LibraryPrep_XT_HS2_ILM protocol requires a bead plate containing 80 µL of beads in each well. Use the AMPureXP_Aliquot (Library Prep) protocol to prepare the bead plate needed for library preparation.

Prepare the NGS Workstation and reagents for the AMPureXP_Aliquot (Library Prep) protocol

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

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

3 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 LibraryPrep_XT_HS2_ILM protocol (each column accommodates 8 gDNA samples).

Load the NGS Workstation

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

Table 16 Initial Bravo deck configuration for AMPureXP_Aliquot (Library Prep) protocol

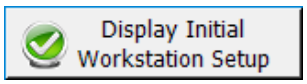
Location	Content
2	New tip box
5	Empty Agilent Deep Well plate
6	Reservoir of AMPure XP bead suspension prepared in **step 3**
8	Empty tip box

Run VWorks protocol AMPureXP_Aliquot (Library Prep)

5 On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot (Library Prep)** protocol.

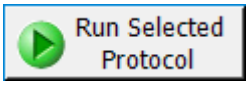
6 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

7 Click **Display Initial Workstation Setup**.



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

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



Running the AMPureXP_Aliquot (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.

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

11 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the **LibraryPrep_XT_HS2_ILM** protocol (refer to **Table 39** on page 63). 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 AMPureXP_XT_HS2_ILM (Pre-Cap PCR) protocols require a bead plate containing 50 µL of beads in each well. Use the AMPureXP_Aliquot (Pre-Capture PCR) protocol to prepare the bead plate needed for purification of pre-capture PCR products.

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

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. 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 (Pre-Cap PCR) protocol (each column accommodates 8 amplified DNA samples).

Load the NGS Workstation

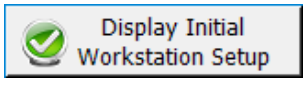
4. Load the Bravo deck according to **Table 17**.

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

Location	Content
2	New tip box
5	Empty Agilent Deep Well Plate
6	Reservoir of AMPure XP bead suspension prepared in **step 3**
8	Empty tip box

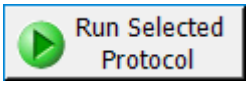
Run VWorks protocol AMPureXP_Aliquot (Pre-Capture PCR)

5. On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot (Pre-Capture PCR)** protocol.
6. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
7. Click **Display Initial Workstation Setup**.



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

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



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

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

11 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the **AMPureXP_XT_HS2_ILM (Pre-Cap PCR)** protocol (refer to **Table 50** on page 72). 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 (Concentration of Pool) protocol is part of the pre-capture pooling workflow. It requires a bead plate containing 180 µL of beads in each well. Use the AMPureXP_Aliquot (Concentration of Pool) protocol to prepare the bead plate needed for concentrating the DNA library pools.

The bead plate for the AMPureXP_XT_HS2_ILM (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 44.

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

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. 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 (Concentration of Pool) protocol (each column accommodates 8 DNA library pools).

Load the NGS Workstation

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

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

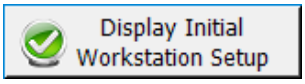
Location	Content
2	New tip box
5	Empty Agilent Deep Well Plate
6	Reservoir of AMPure XP bead suspension prepared in **step 3**
8	Empty tip box

Run VWorks protocol AMPureXP_Aliquot (Concentration of Pool)

5 On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot (Concentration of Pool)** protocol.

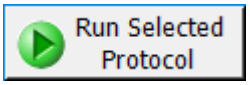
6 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

7 Click **Display Initial Workstation Setup**.



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

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



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

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

11 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the **AMPureXP_XT_HS2_ILM (Concentration of Pool)** protocol (refer to **Table 62** on page 93). 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 AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocol requires a bead plate containing 50 µL of beads in each well. Use the AMPureXP_Aliquot (Post-Capture PCR) protocol to prepare the bead plate needed for purification of post-capture PCR products.

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

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. 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 (Post-Capture PCR) protocol (each column accommodates 8 indexed libraries).

Load the NGS Workstation

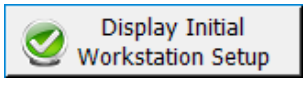
4. Load the Bravo deck according to **Table 19**.

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

Location	Content
2	New tip box
5	Empty Agilent Deep Well Plate
6	Reservoir of AMPure XP bead suspension prepared in **step 3**
8	Empty tip box

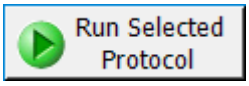
Run VWorks protocol AMPureXP_Aliquot (Post-Capture PCR)

5. On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot (Post-Capture PCR)** protocol.
6. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
7. Click **Display Initial Workstation Setup**.



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

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



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

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

11 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the **AMPureXP_XT_HS2_ILM (Post-Capture PCR)** protocol (refer to **Table 102** on page 136). Use the plate within the next 24 hours.

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

4 Sample Preparation using +/- MBC Adaptors

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

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 9** on **page 163**.

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

Step 1. Prepare and analyze quality of genomic DNA samples

Preparation of high-quality gDNA from fresh biological samples

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

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

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

Additional qualification of DNA samples is not required for DNA derived from fresh biological samples. Depending on the type of sample fragmentation method to be used, proceed to either **"Method 1: Prepare fragmented DNA by enzymatic fragmentation"** on page 50 or **"Method 2. Prepare fragmented DNA by mechanical shearing"** on page 57.

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 20**.

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 20 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 21** to determine the recommended amount of input DNA for the sample.

Table 21 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 44** on page 66. Samples with differing PCR cycle number requirements must be processed in separate library preparation runs.

Step 2. Fragment the DNA

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

- Method 1 uses enzymatic fragmentation, with liquid-handling steps executed by automation protocols. The instructions are described in **"Method 1: Prepare fragmented DNA by enzymatic fragmentation"** on page 50.
- Method 2 uses mechanical shearing, and is described in **"Method 2. Prepare fragmented DNA by mechanical shearing"** on page 57.

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

Method 1: Prepare fragmented DNA by enzymatic fragmentation

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

Method 1 uses the components listed in **Table 22**. Thaw and mix each component as directed in **Table 22** before use. Before starting the run, you need to prepare the Fragmentation master mix (with overage) without the DNA sample. **Table 25** provides Fragmentation master mix volumes for runs that include 1, 2, 3, 4, 6, and 12 columns of samples.

Table 22 Reagents thawed before use in protocol

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
5X SureSelect Fragmentation Buffer (blue cap)	SureSelect Enzymatic Fragmentation Kit, –20°C	Thaw on ice then keep on ice	Vortexing	**page 52**
SureSelect Fragmentation Enzyme (green cap)	SureSelect Enzymatic Fragmentation Kit, –20°C	Place on ice just before use	Inversion	**page 52**

Prepare the NGS Workstation for protocol EnzFrag_XT_HS2_ILM

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 21. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.

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

Pre-program the thermal cycler for the fragmentation reaction

6 Pre-program a thermal cycler using the program in **Table 23**. Start the program, then immediately pause the program.

Table 23 Thermal cycler program for enzymatic fragmentation*

Step	Temperature	Time
Step 1	37°C	Varies -- see **Table 24**
Step 2	65°C	5 minutes
Step 3	4°C	Hold

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

Optimal fragmentation conditions may vary based on the NGS read length to be used in the workflow. Refer to **Table 24** below for the duration at 37°C appropriate for your sample type and required NGS read length.

Table 24 Fragmentation duration based on sample type and NGS read length

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

Prepare the sample plate for fragmentation

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

See **Table 20** or **Table 21** for FFPE DNA input guidelines based on the measured DNA quality in each sample.

Prepare the Fragmentation master mix

8 Prepare the appropriate volume of Fragmentation master mix by combining the reagents in **Table 25**.

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 25 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	43 µL	60 µL	77 µL	98 µL	136 µL	254 µL
5X SureSelect Fragmentation Buffer (blue cap)	2 µL	43 µL	60 µL	77 µL	98 µL	136 µL	254 µL
SureSelect Fragmentation Enzyme (green cap)	1 µL	21 µL	30 µL	38 µL	49µL	68 µL	127 µL
Total Volume	**5 µL**	**107 µL**	**150 µL**	**192 µL**	**245 µL**	**340 µL**	**635 µL**

Prepare the master mix source plate

9 Prepare the **Agilent Deep Well** master mix source plate for the run as indicated in **Table 26**. Add the indicated volume of the master mix to all wells of the indicated column of the Eppendorf plate. Keep the master mix on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 3**.

Table 26 Preparation of the master mix source plate for EnzFrag_XT_HS2_ILM protocol

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Fragmentation master mix	Column 1 (A1-H1)	13 µL	18 µL	23 µL	29 µL	40 µL	75 µL

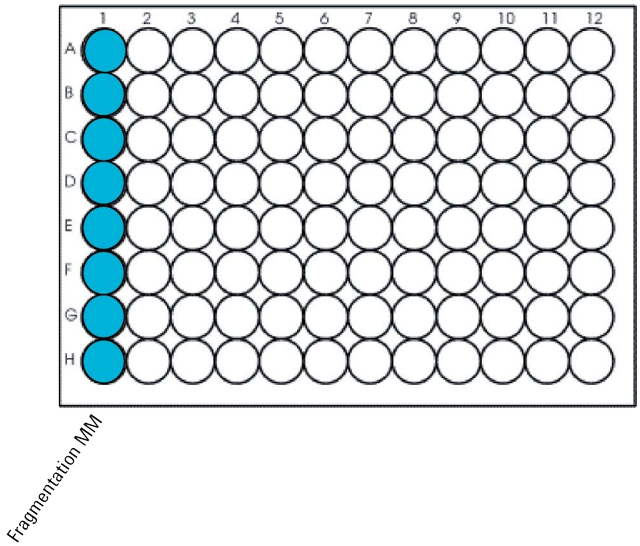


Figure 3 Configuration of the **Agilent Deep Well** master mix source plate for protocol EnzFrag_XT_HS2_ILM

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

11 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

12 Load the Labware MiniHub according to **Table 27**, using the plate orientations shown in **Figure 4**.

Table 27 Initial MiniHub configuration for EnzFrag_XT_HS2_ILM 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

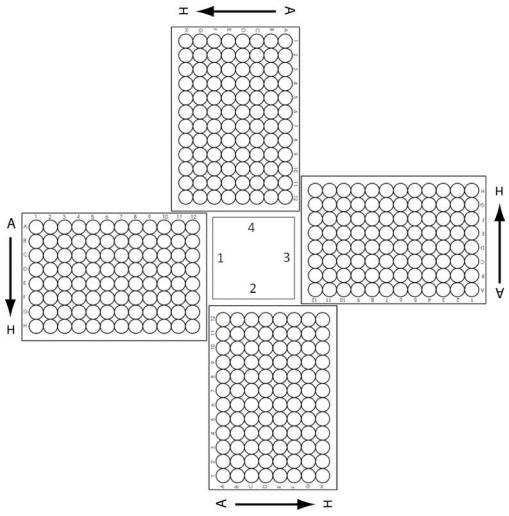


Figure 4 Agilent Labware MiniHub plate orientation.

13 Load the Bravo deck according to **Table 28**.

Table 28 Initial Bravo deck configuration for EnzFrag_XT_HS2_ILM protocol

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

14 Load the BenchCel Microplate Handling Workstation according to **Table 29**.

Table 29 Initial BenchCel configuration for EnzFrag_XT_HS2_ILM 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	—	—	—

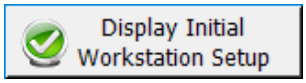
Run VWorks protocol EnzFrag_XT_HS2_ILM

15 On the SureSelect setup form, under **Select protocol to execute**, select the **EnzFrag_XT_HS2_ILM** protocol.

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

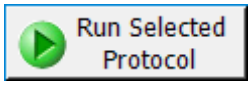
17 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

18 Click **Display Initial Workstation Setup**.



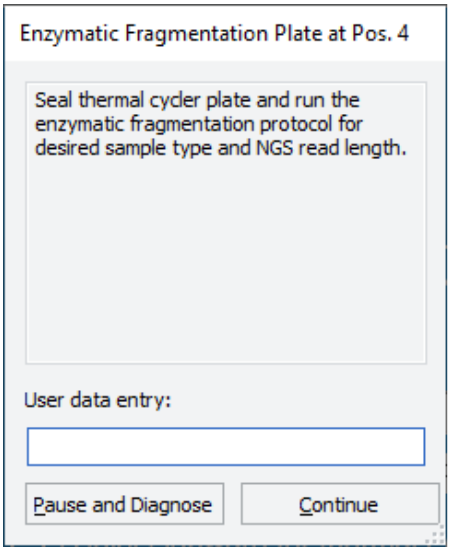
19 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



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

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



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

23 Immediately place the sample plate in the thermal block. Close the lid and resume the thermal cycler program in **Table 23**.

24 From the Bravo deck, remove the Eppendorf twin.tec plate that was used as the Fragmentation master mix source plate from position 9 and set it aside. You will use this same plate again for the Pre-CapPCR_XT_HS2_ILM protocol as described in **"Prepare the pre-capture PCR master mix and master mix source plate"** on page 67.

Prepare the NGS Workstation for protocol EnzFrag_Dil_XT_HS2_ILM

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

26 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.

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

Prepare the sample dilution water reservoir

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

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

At the end of the automation protocol, retain this reservoir for use in the LibraryPrep_XT_HS2_ILM runset and AMPureXP_XT_HS2_ILM (Pre-Cap PCR) protocol.

Load the NGS Workstation

29 Load the Labware MiniHub according to **Table 30**, using the plate orientations shown in **Figure 4** on page 53.

Table 30 Initial MiniHub configuration for EnzFrag_Dil_XT_HS2_ILM protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 28**	—	—
Shelf 1 (Bottom)	—	—	—	—

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

Table 31 Initial Bravo deck configuration for EnzFrag_Dil_XT_HS2_ILM protocol

Location	Content
2	New tip box
4	PCR plate containing fragmented DNA samples seated in red insert (PCR plate type must be specified on setup form under Parameter 2)
5	Empty Eppendorf twin.tec plate, full-skirted (if the PCR plate at position 4 is not a full-skirted Eppendorf twin.tec PCR plate) OR Empty (if the PCR plate at position 4 is a full-skirted Eppendorf twin.tec PCR plate)
8	Empty tip box

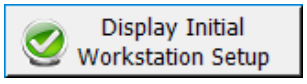
Run VWorks protocol EnzFrag_Dil_XT_HS2_ILM

31 On the SureSelect setup form, under **Select protocol to execute**, select the **EnzFrag_Dil_XT_HS2_ILM** protocol.

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

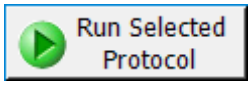
33 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

34 Click **Display Initial Workstation Setup**.



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

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



Running the EnzFrag_Dil_XT_HS2_ILM protocol takes approximately 5 minutes. Once complete, the samples are ready for library preparation. The samples are located in the Eppendorf plate at position 7 of the Bravo deck.

NOTE

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

Proceed directly to **"Step 3. Prepare adaptor-ligated libraries"** on page 59.

Method 2. Prepare fragmented DNA by mechanical shearing

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

The target fragment size and corresponding shearing conditions may vary for workflows using different NGS read lengths. See **Table 32** for guidelines. Complete shearing instructions are provided on **page 58**.

Table 32 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 20** or **Table 21** 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 33**.

Table 33 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 a Eppendorf twin.tec 96-well plate sample well. 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**.

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. Prepare adaptor-ligated libraries

In this step, the NGS Workstation completes the DNA end modification steps required for SureSelect target enrichment, including end-repair, dA-tailing, and ligation of the adaptors (with or without MBCs). After the end-modification steps, the NGS Workstation purifies the prepared DNA using AMPure XP beads.

This step uses the components listed in **Table 34**. Thaw and mix each component as directed in **Table 34** before use. Before starting the run, you need to prepare master mixes (with overage) for each step, without the DNA sample. Master mixes for runs that include 1, 2, 3, 4, 6, and 12 columns (including overage) are shown in reagent preparation tables.

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

Table 34 Reagents thawed before use in protocol

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
End Repair-A Tailing Buffer (bottle)	**For MBC-tagged libraries**, get reagents from the SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR) box, stored at –20°C **For MBC-free libraries**, get reagents from the SureSelect XT HS2 Library Preparation Kit for ILM, MBC-Free (Pre PCR) box, stored at –20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	**page 60**
Ligation Buffer (bottle)		Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	**page 61**
End Repair-A Tailing Enzyme Mix (orange cap)		Place on ice just before use	Inversion	**page 60**
T4 DNA Ligase (blue cap)		Place on ice just before use	Inversion	**page 61**
XT HS2 Adaptor Oligo Mix (white cap) -- OR -- SureSelect MBC-Free Adaptor Oligo Mix (black cap)		Thaw on ice then keep on ice	Vortexing	**page 61**

Prepare the NGS Workstation

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Pre-set the temperature of Bravo deck position 4 to 79°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
3. Pre-set the temperature of Bravo deck position 6 to 20°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. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.

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

1 Prepare the appropriate volume of End Repair/dA-Tailing master mix, using volumes listed in **Table 35** 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 35 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

1 Prepare the appropriate volume of Ligation master mix, using volumes listed in **Table 36** 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 36 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 µL	489 µL	684 µL	880 µL	1271 µL	2737 µL
T4 DNA Ligase (blue cap)	2 µL	26 µL	43 µL	60 µL	77 µL	111 µL	238 µL
Total Volume	**25 µL**	**319 µL**	**532 µL**	**744 µL**	**956 µL**	**1381 µL**	**2975 µL**

Prepare the Adaptor Oligo Mix

1 Prepare the appropriate volume of Adaptor Oligo Mix dilution, according to **Table 37**. Mix well using a vortex mixer and keep on ice.

Table 37 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	43 µL	64 µL	85 µL	106 µL	144 µL	276 µL
XT HS2 Adaptor Oligo Mix (white cap) -- OR -- SureSelect MBC-Free Adaptor Oligo Mix (black cap)	5 µL	85 µL	128 µL	170 µL	213 µL	287 µL	553 µL
Total Volume	**7.5 µL**	**128 µL**	**191 µL**	**255 µL**	**319 µL**	**431 µL**	**829 µL**

Prepare the master mix source plate

1 Prepare the **Agilent Deep Well** master mix source plate containing the mixtures prepared in **step 1** through **step 1**. Add the volumes indicated in **Table 38** of each mixture to all wells of the indicated column of the Agilent Deep Well plate. Keep the master mixes on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 5**.

Table 38 Preparation of the master mix source plate for LibraryPrep_XT_HS2_ILM 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
End Repair-dA Tailing master mix	Column 2 (A2-H2)	31 µL	52 µL	73 µL	94 µL	136 µL	280 µL
Ligation master mix	Column 3 (A3-H3)	36 µL	62 µL	88 µL	114 µL	166 µL	360 µL
Adaptor Oligo Mix dilution	Column 4 (A4-H4)	15 µL	23 µL	30 µL	38 µL	53 µL	101 µL

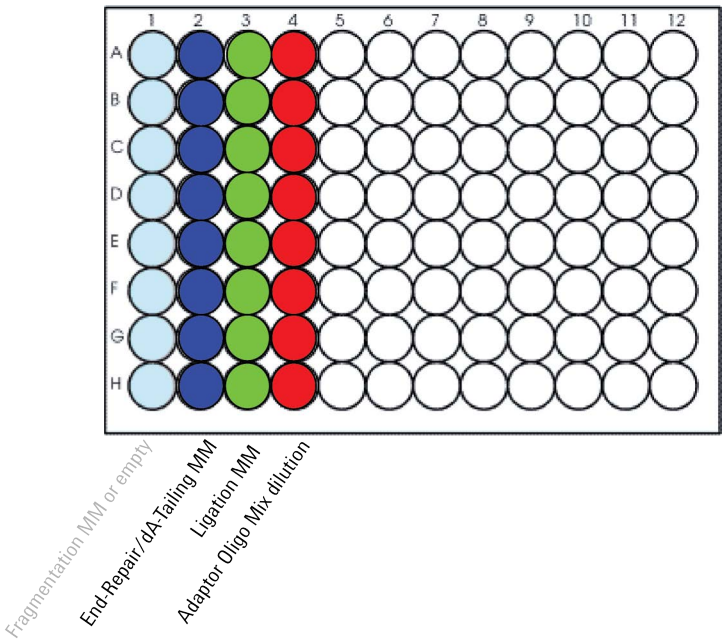


Figure 5 Configuration of the **Agilent Deep Well** master mix source plate for runset LibraryPrep_XT_HS2_ILM. The master mix dispensed during the EnzFrag_XT_HS2_ILM protocol (if used) is shown in light shading.

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

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

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

Prepare the purification reagents

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

Use the same Agilent shallow well reservoir that was used in the EnzFrag_Dil_XT_HS2_ILM protocol (if the Enzymatic Fragmentation method was used for shearing) or use a fresh Agilent shallow well reservoir.

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

At the end of the automation protocol, retain this reservoir for use in the AMPureXP_XT_HS2_ILM (Pre-Cap PCR) protocol.

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

Load the NGS Workstation

1 Load the Labware MiniHub according to **Table 39**, using the plate orientations shown in **Figure 4** on page 53.

Table 39 Initial MiniHub configuration for LibraryPrep_XT_HS2_ILM runset

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Aliquotted AMPure XP beads in Agilent deep well plate from **page 38** (80 µL of beads/well)	—	—	—
Shelf 4	Empty Eppendorf twin.tec plate	—	—	—
Shelf 3	Empty Eppendorf twin.tec plate	Empty Eppendorf twin.tec plate	—	—
Shelf 2	New tip box (or box from protocol EnzFrag_XT_HS2_ILM)	Nuclease-free water reservoir from **step 1**	—	—
Shelf 1 (Bottom)	Empty tip box (or box from protocol EnzFrag_XT_HS2_ILM)	70% ethanol reservoir from **step 2**	—	Empty tip box

2 Load the Bravo deck according to **Table 40**.

Table 40 Initial Bravo deck configuration for LibraryPrep_XT_HS2_ILM runset

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	Empty red insert
6	Empty Eppendorf twin.tec plate
7	Eppendorf plate containing sheared gDNA samples
9	Library Prep master mix source plate, unsealed

3 Load the BenchCel Microplate Handling Workstation according to **Table 41**.

Table 41 Initial BenchCel configuration for LibraryPrep_XT_HS2_ILM runset

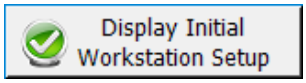
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	2 Tip boxes	—	—	—
3	2 Tip boxes	—	—	—
4	3 Tip boxes	—	—	—
6	4 Tip boxes	—	—	—
12	7 Tip boxes	—	—	—

Run VWorks runset LibraryPrep_XT_HS2_ILM

4 On the SureSelect setup form, under **Select protocol to execute**, select the **LibraryPrep_XT_HS2_ILM** runset.

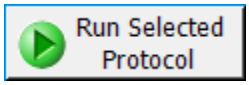
5 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

6 Click **Display Initial Workstation Setup**.

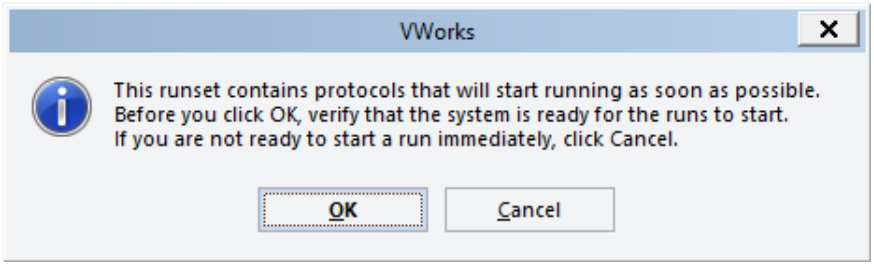


7 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



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



Running the LibraryPrep_XT_HS2_ILM runset takes approximately 2 hours. Once complete, the purified, adaptor-ligated DNA samples are located in the Eppendorf plate at position 7 of the Bravo deck.

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

Step 4. Amplify adaptor-ligated libraries

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

This step uses the components listed in **Table 42**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 42 Reagents for pre-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Pipette up and down 15–20 times	**page 67**
5× Herculase II Reaction Buffer (clear cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	**page 67**
SureSelect XT HS2 Index Primer Pairs	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR),* –20°C	Vortexing	**page 67**

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

CAUTION

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

The SureSelect XT HS2 Index Primer Pairs are provided in single-use aliquots. To avoid cross-contamination of libraries, do not retain and re-use any residual volume in the plate for subsequent experiments.

Prepare the NGS Workstation

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

Pre-program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 43**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the automation run.

Table 43 Pre-Capture PCR Thermal Cycler Program*

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8 to 14, based on input DNA quality and quantity (see **Table 44**)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

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

Table 44 Pre-capture PCR cycle number recommendations

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

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

Prepare the SureSelect XT HS2 Index Primer Pairs

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 the PCR plate to be used for the pre-capture PCR thermal cycling. Make sure to maintain the same well location for each primer pair when transferring to the PCR plate. Keep the PCR plate on ice.

The PCR plate containing the primer pairs is loaded onto the Bravo deck in **step 2** on **page 69** for the Pre-CapPCR_XT_HS2_ILM protocol.

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

1 Prepare the appropriate volume of pre-capture PCR master mix, according to **Table 45**. Vortex at medium speed for 15–20 seconds and keep on ice.

Table 45 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	340 µL	425 µL	574 µL	1066 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17 µL	26 µL	34 µL	43 µL	57 µL	107 µL
Total Volume	**11 µL**	**187 µL**	**281 µL**	**374 µL**	**468 µL**	**631 µL**	**1173 µL**

2 Using the same **Agilent Deep Weel** master mix source plate that was used for the LibraryPrep_XT_HS2_ILM protocol, add the volume of PCR master mix indicated in **Table 46** to all wells of column 5 of the master mix source plate. The final configuration of the master mix source plate is shown in **Figure 6**.

Table 46 Preparation of the master mix source plate for Pre-CapPCR_XT_HS2_ILM protocol

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Pre-Capture PCR Master Mix	Column 5 (A5-H5)	22 µL	33 µL	44 µL	55 µL	77 µL	143 µL

CAUTION Make sure to add the Pre-Capture PCR master mix to column 5 of the source plate.

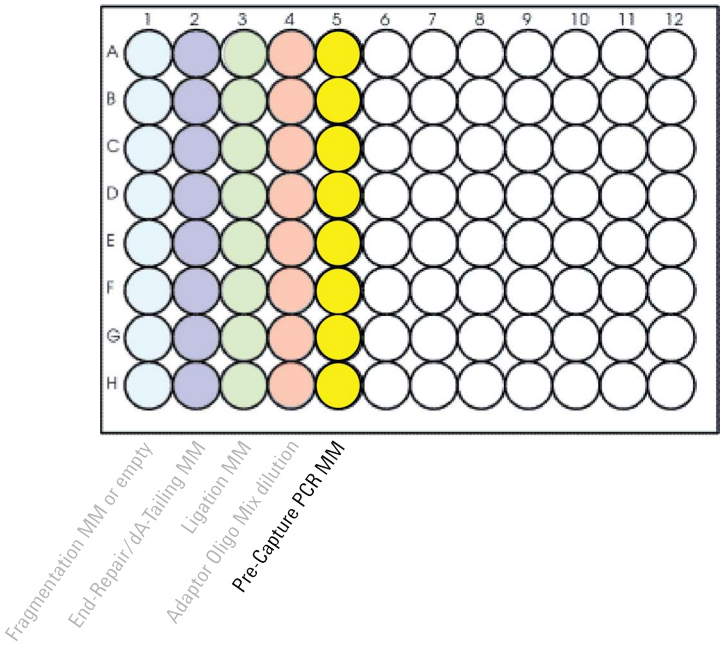


Figure 6 Configuration of the **Agilent Deep Well** master mix source plate for protocol Pre-CapPCR_XT_HS2_ILM. Master mixes dispensed during previous protocols are shown in light shading.

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

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

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 47**, using the plate orientations shown in **Figure 4** on page 53.

Table 47 Initial MiniHub configuration for Pre-CapPCR_XT_HS2_ILM protocol

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

2 Load the Bravo deck according to **Table 48**.

Table 48 Initial Bravo deck configuration for Pre-CapPCR_XT_HS2_ILM protocol

Location	Content
6	SureSelect XT HS2 Index Primer Pairs for ILM in PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
7	Adaptor-ligated DNA samples in Eppendorf twin.tec plate
9	Master mix plate containing PCR master mix in column 2 (unsealed)

3 Load the BenchCel Microplate Handling Workstation according to **Table 49**.

Table 49 Initial BenchCel configuration for Pre-CapPCR_XT_HS2_ILM 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	—	—	—

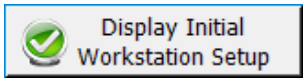
Run VWorks protocol Pre-CapPCR_XT_HS2_ILM

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

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

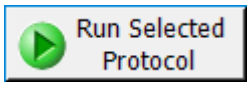
3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

4 Click **Display Initial Workstation Setup**.



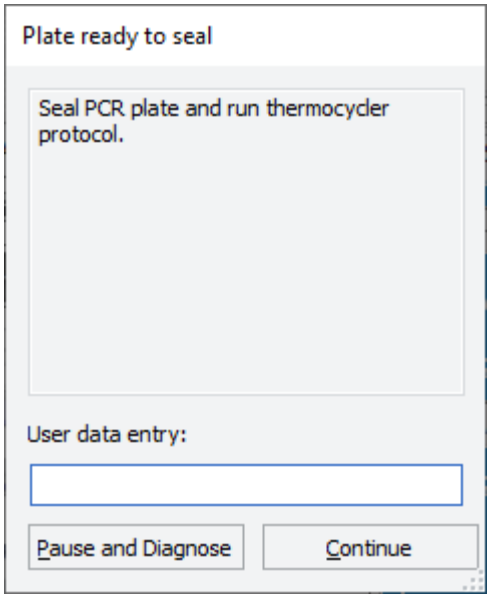
5 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



Running the Pre-CapPCR_XT_HS2_ILM protocol takes approximately 15 minutes. Once complete, the PCR-ready samples, containing prepped DNA and PCR master mix, are located in the PCR plate at position 6 of the Bravo deck.

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



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

9 Before adding the samples to the pre-programmed thermal cycler, bring the temperature of the thermal block to 98°C by resuming the thermal cycler program in **Table 43**. Once the cycler has reached 98°C, immediately place the sample plate in the thermal block and close the lid.

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

Step 5. Purify amplified DNA using AMPure XP beads

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

This step uses the aliquoted plate of AMPure XP beads that was prepared on **page 40**.

Prepare the NGS Workstation and reagents

1. Retain the Eppendorf twin.tec master mix source plate containing the Pre-Capture PCR master mix located at position 9 of the Bravo deck for later use in the TS_D1000 protocol (see **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape"** on page 74). Otherwise, clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 45°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
5. Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water. Use the same Agilent shallow well reservoir that was used in the EnzFrag_XT_HS2_ILM protocol and LibraryPrep_XT_HS2_ILM runset.

   Make sure that the water in the reservoir does not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.
6. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.
7. Load the Labware MiniHub according to **Table 50**, using the plate orientations shown in **Figure 4** on page 53.

Table 50 Initial MiniHub configuration for AMPureXP_XT_HS2_ILM (Pre-Cap PCR) protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Aliquotted AMPure XP beads in Agilent Deep Well plate from **page 40** (50 µL of beads/well)	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	Empty Eppendorf twin.tec Plate	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 5**		—
Shelf 1 (Bottom)	—	70% ethanol reservoir from **step 6**	—	Empty tip box

8 Load the Bravo deck according to **Table 51**.

Table 51 Initial Bravo deck configuration for AMPureXP_XT_HS2_ILM (Pre-Cap PCR) protocol

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

9 Load the BenchCel Microplate Handling Workstation according to **Table 52**.

Table 52 Initial BenchCel configuration for AMPureXP_XT_HS2_ILM (Pre-Cap PCR) protocol

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

Run VWorks protocol AMPureXP_XT_HS2_ILM (Pre-Cap PCR)

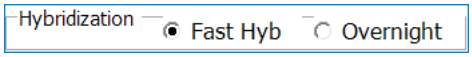
1 On the SureSelect setup form, under **Select protocol to execute**, select one of the AMPureXP_XT_HS2_ILM (Pre-Cap PCR) protocols based on your workflow of choice.

- If you are using the post-capture pooling workflow (i.e., pooling samples after hybridization with the Probe) then select the protocol **AMPureXP_XT_HS2_ILM (Case Pre-Cap PCR - SinglePlex)**.
- If you are using the pre-capture pooling workflow (i.e., pooling samples prior to hybridization with the Probe) then select the protocol **AMPureXP_XT_HS2_ILM (Case Pre-Cap PCR - MultiPlex)**.

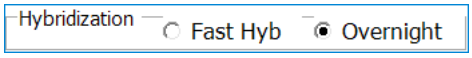
Selecting the correct option is important for the downstream hybridization protocol.

2 Select the correct **Hybridization** option based on your hybridization method.

- If you will be performing fast hybridization, select **Fast Hyb**.



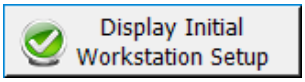
- If you will be performing overnight hybridization, select **Overnight**.



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

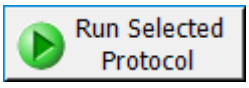
4 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

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**.



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

Step 6. 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 74.
- 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 80.

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. Clear the Labware MiniHub and BenchCel of plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn off the ThermoCube device (see **page 22**) to restore position 9 of the Bravo deck to room temperature.
4. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
5. Using an **Eppendorf twin.tec** source plate, prepare the Sample Buffer source plate at room temperature. Add the volume of D1000 Sample Buffer indicated in **Table 53** to each well of column 1 of the plate.

Table 53 Preparation of the Sample Buffer source plate for TS_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer 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
D1000 Sample Buffer	Column 1 (A1-H1)	11 µL	17 µL	23 µL	29 µL	41 µL	77 µL

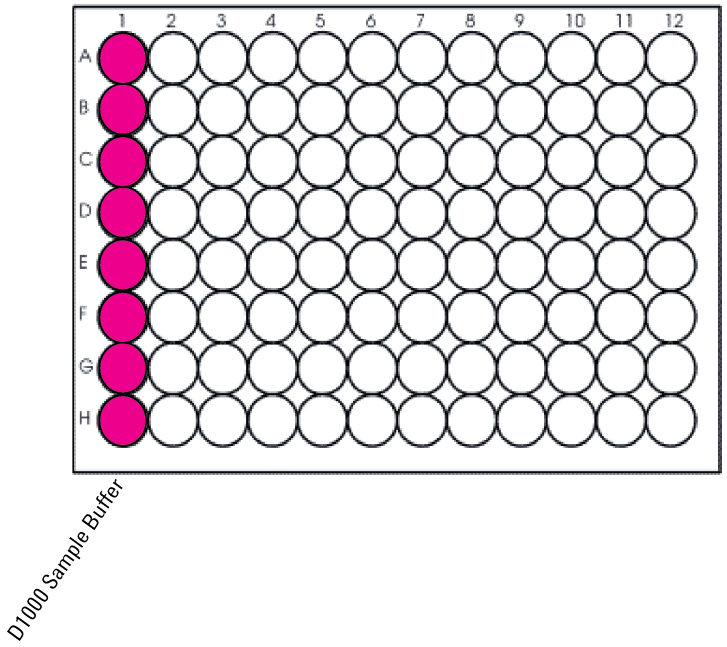


Figure 7 Configuration of the **Eppendorf twin.tec** source plate for protocol TS_D1000

Load the NGS Workstation

6 Load the Labware MiniHub according to **Table 54**, using the plate orientations shown in **Figure 4** on page 53.

Table 54 Initial MiniHub configuration for TS_D1000 protocol

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

7 Load the Bravo deck according to **Table 55**.

Table 55 Initial Bravo deck configuration for TS_D1000 protocol

Location	Content
4	Amplified pre-capture libraries in Eppendorf twin.tec plate (unsealed)
6	Empty Eppendorf twin.tec plate
9	Eppendorf twin.tec source plate containing D1000 Sample Buffer in Column 3

CAUTION

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

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

8 Load the BenchCel Microplate Handling Workstation according to **Table 56**.

Table 56 Initial BenchCel configuration for TS_D1000 protocol

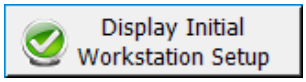
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Run VWorks protocol TS_D1000

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

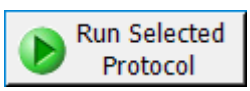
10 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

11 Click **Display Initial Workstation Setup**.



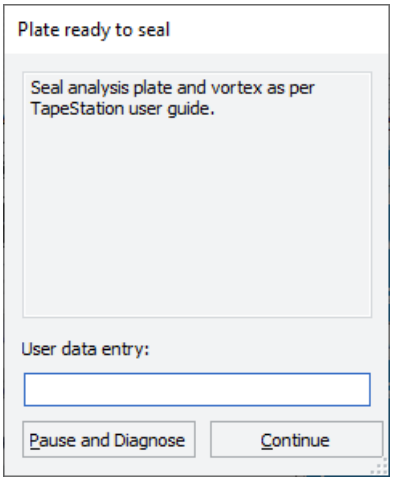
12 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



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

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



CAUTION

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

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

Run the D1000 Assay and analyze the data

15 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.

16 Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 57** for guidelines). Sample electropherograms are shown in **Figure 8** (library prepared from high-quality DNA), **Figure 9** (library prepared from medium-quality FFPE DNA), and **Figure 10** (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 10**. See Troubleshooting information on **page 195** for additional considerations.

Table 57 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

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

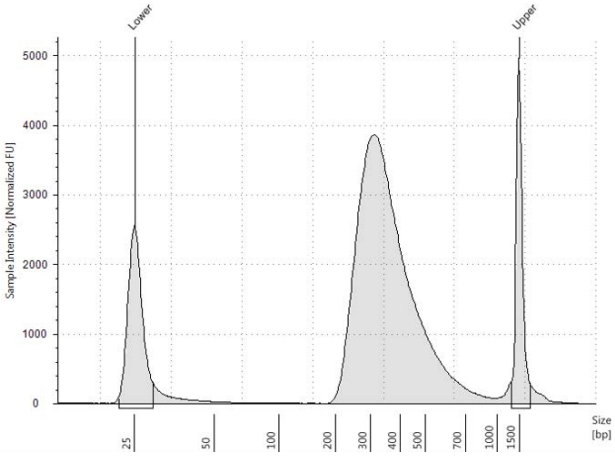


Figure 8 Pre-capture library prepared from a high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

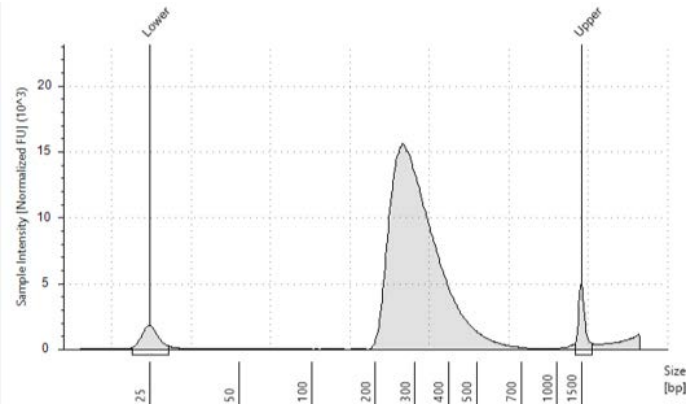


Figure 9 Pre-capture library prepared from a typical FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

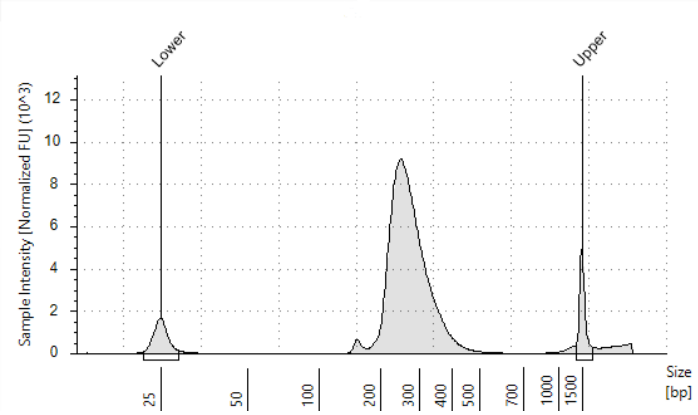


Figure 10 Pre-capture library prepared from a low-quality FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

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 8** through **Figure 10**). **Table 58**. Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 57** for guidelines). **Table 58** includes links to assay instruction.

Table 58 Pre-capture library analysis options

Analysis platform	Assay used at this step	Link to assay instructions	Amount of library sample to analyze
Agilent 4200/4150 TapeStation system	D1000 ScreenTape	**Agilent D1000 Assay Quick Guide**	1 µL of sample mixed with 3 µL of D1000 sample buffer
Agilent 2100 BioAnalyzer system	DNA 1000 Kit	**Agilent DNA 1000 Kit Guide**	1 µL of sample
Agilent 5200, 5300, or 5400 Fragment Analyzer system	NGS Fragment Kit (1-6000 bp)	**Agilent NGS Fragment Kit (1-6000 bp) Guide**	2 µL of sample

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

5 Hybridization (Fast)

This chapter describes the steps to hybridize the DNA library or library pool to the Probe using the fast hybridization method. The DNA libraries are hybridized to a SureSelect or ClearSeq Probe using single-plex or multi-plex hybridization in a thermal cycler program in which the hybridization segment is 1 to 2 hours.

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 82.
- 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 85.

NOTE For instructions on the overnight hybridization method, see **Chapter 6**, "Hybridization (Overnight)."

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

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 85.

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 73** to **page 80**, 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 minimum of 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 for hybridization. Before running the automation protocol, you must create a table containing instructions for the NGS Workstation indicating the volume of each sample to aliquot, as described in the steps below.

1. Create a .csv (comma separated value) file with the headers shown in **Figure 11**. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in .csv format. The file must include rows for all 96 wells of the plate.
2. Enter the information requested in the header for each DNA sample.
   - In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
   - In the SourceWell and DestinationWell fields, enter each well position for the plate. SourceWell and DestinationWell field contents must be identical for a given sample.
   - In the Volume field, enter the volume (in µL) of each DNA sample to be used in the hybridization step (see **page 82** for guidelines). For all empty wells on the plate, delete the corresponding rows in the .csv file.

	A	B	C	D
1	SourceBC	SourceWell	DestinationWell	Volume
2	abc	A1	A1	5.35
3	abc	B1	B1	4.28
4	abc	C1	C1	5.19
5	abc	D1	D1	4.76
6	abc	E1	E1	5.19
7	abc	F1	F1	5.49
8	abc	G1	G1	4.86
9	abc	H1	H1	5.05
10	abc	A2	A2	4.37

Figure 11 Sample spreadsheet for 1-column run

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Aliquot Input File Templates\Aliquot_Libraries_Template.csv**.

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

3 Load the .csv file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Aliquot Input File Templates**.

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.

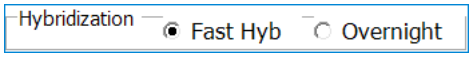
5 Load the Bravo deck according to Table 59.

Table 59 Initial Bravo deck configuration for Aliquot_Libraries protocol

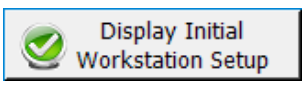
Location	Content
2	Nuclease-free water in Agilent shallow well reservoir (30 mL)
5	Empty Eppendorf twin.tec plate
6	Empty tip box
8	New tip box
9	Prepped library DNA in Eppendorf twin.tec plate

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

7 Select the **Fast hyb** option.



8 Click **Display Initial Workstation Setup**.



9 Upload the .csv file created in **step 1** through **step 3** on **page 82**.

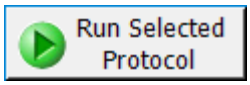
a Click the "**...**" browse button below **Select Aliquot Input File** to open a directory browser window.

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

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

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 5.

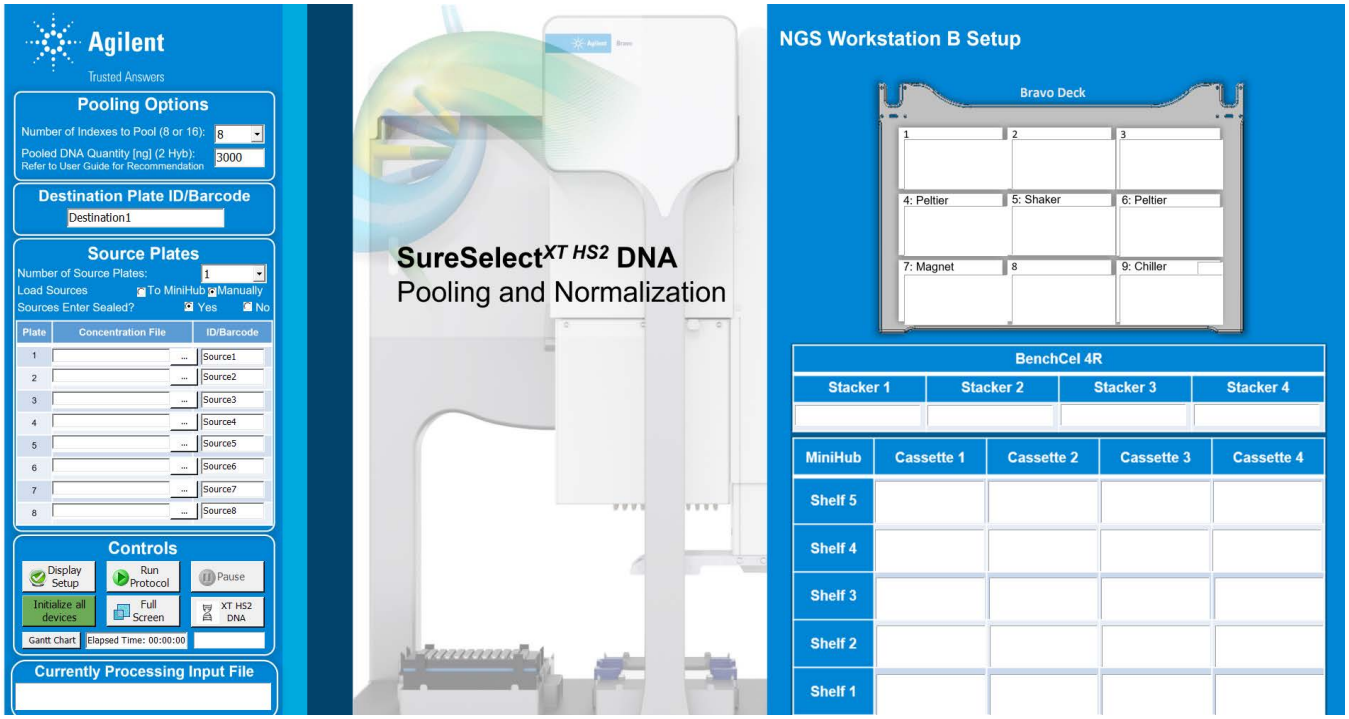
12 Remove the sample plate from the Bravo deck.

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 82.

Pool indexed DNA samples for hybridization

In this step, the NGS Workstation pools the prepped indexed gDNA samples before hybridization to the Probe. This workflow step is set up using the VWorks Form XT_HS2_Pooling.VWForm shown below, which is accessible from within the XT_HS2_ILM form.



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 60** for the recommended pool composition based on your SureSelect Probe.

Table 60 Pre-capture pooling of indexed DNA libraries

Probe	Amount of total DNA per pool (Amount of DNA pool per hybridization reaction)*	Number of indexed gDNA libraries per pool	Amount of each indexed gDNA library in pool	Maximum DNA concentration for pool
SureSelect XT HS PreCap Human All Exon V8/V8+UTR/V8+NCV or SureSelect Clinical Research Exome V4	6000 ng (3000 ng/hybridization)	8	750 ng	375 ng/µL
SureSelect XT HS PreCap Human All Exon V7	3000 ng (1500 ng/hybridization)	8	375 ng	187.5 ng/µL
SureSelect PreCap Custom Probes	3000 ng (1500 ng/hybridization)	16	187.5 ng	93.75 ng/µL

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

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

Accurate normalization of pools requires a minimum pipetting volume of 2 µL for each sample. Maximum DNA concentration values for a pool containing >2 µL of each sample are shown in **Table 60**, above. When higher-concentration DNA samples are included in the pooling run, the DNA pool amount must be adjusted as described below.

- Check the DNA concentration of each sample in the set of source plates to be pooled to a single destination plate to determine the appropriate amount of DNA per pool.
  - If all samples contain DNA at concentrations below the maximum DNA concentration shown in **Table 60**, 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 60**).
  - If at least one of the samples is above the maximum DNA concentration shown in **Table 60**, then you need to calculate the appropriate DNA pool amount. First, identify the most concentrated DNA sample and calculate the amount of DNA contained in 2 µL of that sample. This becomes the amount of each DNA sample used for pooling in the run. For example, if the highest DNA sample concentration is 200 ng/µL, then the final DNA pool will contain 400 ng of each indexed DNA. Next, determine the total amount of DNA per pool, based on the Probe size. Continuing with the same example, a Focused Exome capture pool would contain 8 × 400 ng, or 3200 ng DNA.

Plan destination indexed DNA pool sample plate configuration

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

- When using a single Probe for all wells on the plate, fill the plate column-wise in well order A1 to H1, then A2 to H2, ending with A12 to H12.
- When using multiple Probes, configure the plate such that all gDNA library pools to be hybridized to a particular Probe are positioned in appropriate rows. When preparing for the Hyb_XT_HS2_ILM protocol, place samples to be enriched using the same Probe in the same row.
- Each 96-reaction library preparation run produces 6 or 12 gDNA pools. For greatest efficiency of reagent use, gDNA pools from multiple library preparation runs may be placed on the same destination plate for hybridization.

Prepare .csv files for pooling and normalization

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

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

C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Pooling and Normalization Templates

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

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

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

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

Figure 12 Sample pooling and normalization .csv file content

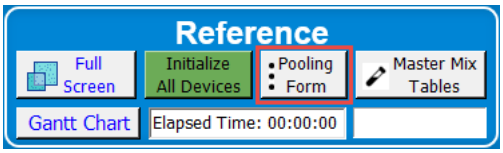
2 In each .csv file, edit the information for each DNA sample (well ID) as follows:
   - In the **PreCap Amplified pond concentrations** field, enter the concentration (in ng/µL) determined on **page 73** for each indexed DNA sample.
   - In the **Target WellID** field, enter the well position of the pool in which the indexed DNA sample should be included for the destination plate. See the guidelines on **page 86** for hybridization sample pool placement considerations.

Set up and run the PreCapture_Pooling automation protocol

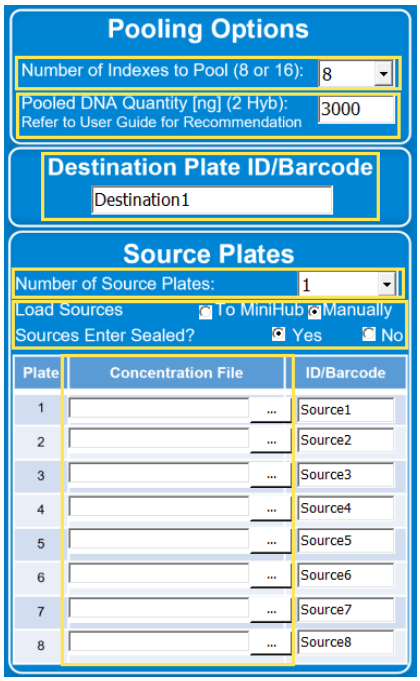
3 Turn on the chiller, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.

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

5 To set up the PreCapture_Pooling automation protocol, open the VWorks Form XT2_HS2_Pooling using one of the methods below.
   - Double-click the shortcut on your desktop for the XT2_HS2_Pooling VWorks Form.
   - In the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Forms** open the file **XT_HS2_Pooling_v.B1.0.VWForm**.
   - From the XT_HS2_ILM VWorks Form, under **Reference**, click **Pooling Form.**



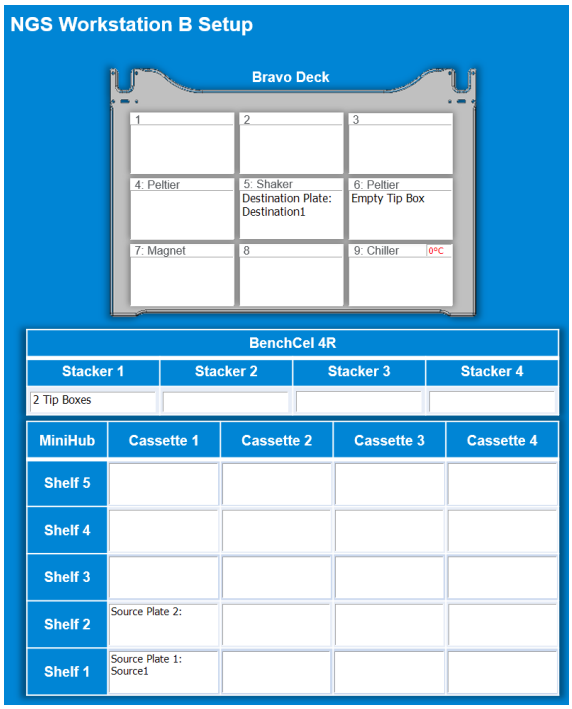
6 In the XT2_HS2_Pooling Form, enter the run information highlighted below:
   - Under **Pooling Options**, from **Number of Indexes to Pool** menu, select 8 or 16 (see **Table 60** on page 86 for guidelines).
   - Under **Pooling Options**, from **Pooled DNA Quantity** menu, enter the required total amount of DNA in the pool.
     - The required amount is either 3000 ng or 6000 ng, depending on the Probe (see **Table 60** on page 86). These amounts are sufficient for two hybridization reactions.
   - Under **Destination Plate ID/Barcode**, enter the name or barcode of the destination plate into the field provided.
   - Under **Source Plates**, from **Number of Source Plates** menu, select the number of indexed DNA source plates to be provided for sample pooling. If >8 plates will be used to create a single hybridization sample plate, run the pooling and normalization protocol in sets of 8 source plates.
   - Under **Source Plates**, specify whether the indexed DNA source plates will be loaded in the MiniHub and will be sealed at start of run (recommended).
   - In the table under **Source Plates**, in the **Concentration File** field, use the browse button to specify the location of each .csv file that provides sample position and concentration data for each plate.



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

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

- Load each indexed DNA source plates onto its assigned shelf on the MiniHub.
- Load an Eppendorf twin.tec destination plate 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.



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

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

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

10 Remove the destination plate from Bravo deck position 5.

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

Adjust final concentration of pooled DNA

Prior to hybridization with the Probe, use the Aliquot_Water and AMPureXP_XT_HS2_ILM (Concentration of Pool) automation protocols to concentrate each DNA library pool to 24 µL, a volume sufficient for two hybridization reactions. First, the Aliquot_Water protocol adds enough water to each DNA library pool to bring the volume to 100 µL. Then, the AMPureXP_XT_HS2_ILM protocol uses AMPure XP beads to purify the DNA library pools, eluting the DNA in a volume of 24 µL.

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

Prepare .csv file for normalizing sample volumes to 100 µL

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

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

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

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

Figure 13 Sample Aliquot_Water .csv file content

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Aliquot Input File Templates**.

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

3 Load the .csv file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\XT_HS2_ILM_v.B1.1.2\Pooling and Normalization Templates**.

Set up and run the Aliquot_Water automation protocol

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

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

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

At the end of the automation protocol, retain this reservoir for use in the SSELCapture&Wash_XT_HS2 runset and Post-CapPCR_XT_HS2_ILM and AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocols, provided you are performing these steps before the end of the day.

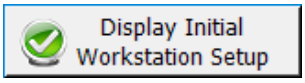
6 Load the Bravo deck according to **Table 61**.

Table 61 Initial Bravo deck configuration for Aliquot_Water protocol

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

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

8 Click **Display Initial Workstation Setup**.



9 Upload the .csv file created in **step 1** through **step 3** on **page 90**.

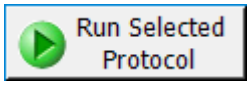
a Click the "**...**" browse button below **Select Aliquot Input File** to open a directory browser window.

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

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

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

12 Remove the sample plate from the Bravo deck.

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

Set up and run the AMPureXP_XT_HS2_ILM (Concentration of Pool) automation protocol

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

15 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.

16 Pre-set the temperature of Bravo deck position 4 to 45°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.

17 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.

18 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.

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

20 Load the Labware MiniHub according to **Table 62**, using the plate orientations shown in **Figure 4** on page 53.

Table 62 Initial MiniHub configuration for AMPureXP_XT_HS2_ILM (Concentration of Pool) protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Aliquotted AMPure XP beads in Agilent deep well plate from **page 42** (180 µL of beads/well)	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	Empty Eppendorf twin.tec Plate	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 18**		—
Shelf 1 (Bottom)	—	70% ethanol reservoir from **step 19**	—	Empty tip box

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

Table 63 Initial Bravo deck configuration for AMPureXP_XT_HS2_ILM (Concentration of Pool) protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
9	Eppendorf twin.tec plate containing DNA library pools from the Aliquot_Water protocol seated in red insert

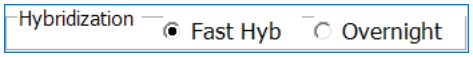
22 Load the BenchCel Microplate Handling Workstation according to **Table 64**.

Table 64 Initial BenchCel configuration for AMPureXP_XT_HS2_ILM (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	—	—	—

23 On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_XT_HS2_ILM (Concentration of Pool)** protocol.

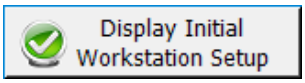
24 Select the **Fast hyb** option.



25 Under **Select labware for thermal cycling**, select **96 Eppendorf Twin.tec PCR in Red Alum Insert**.

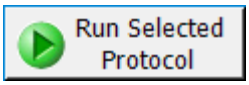
26 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

27 Click **Display Initial Workstation Setup**.



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

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



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

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

In this step, automation protocol Hyb_XT_HS2_ILM is used to complete the liquid handling steps to set up the hybridization reactions. Afterward, you transfer the sample plate to a thermal cycler, held at 65°C, to allow hybridization of the DNA samples to the probe.

This step uses the components listed in **Table 65**. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 65 Reagents for Hybridization

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect XT HS2 Blocker Mix (blue cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), stored at –20°C	Thaw on ice	**page 98**
SureSelect RNase Block (purple cap)		Thaw on ice	**page 98**
SureSelect Fast Hybridization Buffer (bottle)		Thaw and keep at Room Temperature	**page 98**
Probe	–80°C	Thaw on ice	**page 98**

Program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with either the program in **Table 66** or the program in **Table 67**, depending on the Probe. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

- **Table 66** – Use this program if you are using one of the SureSelect XT HS Human All Exon V8 Probes (V8, V8+UTR, or V8+NCV) or the SureSelect XT HS CRE V4 Probe. These Probes require a longer hybridization on the thermal cycler.
- **Table 67** – Use this program if you are NOT using one of the Probes mentioned above.

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

Table 66 Fast hybridization program for the *SureSelect XT HS Human All Exon V8 Probes* and *SureSelect XT HS CRE V4 Probe**

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

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

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

‡ Samples are held at 65°C until they are processed in the Capture & Wash automation protocol that begins on **page 130**.

Table 67 Fast hybridization program for *all other probes**

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

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

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

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

** Samples are held at 65°C until they are processed in the Capture & Wash automation protocol that begins on **page 130**.

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

NOTE

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

- In the final segment of the thermal cycler program (**Table 66** or **Table 67**), replace the final brief 65°C Hold step with a 21°C Hold step.
- The hybridized samples may be held at 21°C for up to 16 hours. Begin the capture preparation steps on **page 130** on day 2 after the overnight hold.

Prepare the NGS Workstation

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

3 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.

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 Place red PCR plate inserts at Bravo deck positions 4 and 6.

6 Place an empty tip box on shelf 1 of cassette 4 of the MiniHub.

7 Load tip boxes for the run in the BenchCel Microplate Handling Workstation according to **Table 68**.

Table 68 Initial BenchCel configuration for Hyb_XT_HS2_ILM protocol

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

Prepare the Block master mix

8 Prepare the appropriate volume of Block master mix, on ice, as indicated in **Table 69**. Mix by vortexing at medium speed for 15–20 seconds. Keep on ice.

Table 69 Preparation of Block master mix for fast hybridization

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	32 µL	53 µL	74 µL	96 µL	138 µL	276 µL
SureSelect XT HS2 Blocker Mix (blue cap)	5.0 µL	64 µL	106 µL	149 µL	191 µL	276 µL	553 µL
Total Volume	**7.5 µL**	**96 µL**	**159 µL**	**223 µL**	**287 µL**	**414 µL**	**829 µL**

Prepare one or more Probe Hybridization master mixes

9 Prepare the appropriate volume of Probe Hybridization master mix for each of the Probes that will be used for hybridization as indicated in **Table 70** to **Table 73**. 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 70** or **Table 71**) on **page 98**.

For runs that use different Probes for individual rows, prepare each master mix as described in Step b (**Table 72** or **Table 73**) on **page 99**.

a For runs that use a single Probe for all rows, prepare a master mix as described in **Table 70** or **Table 71**, according to the probe design size.

Table 70 Preparation of Probe Hybridization master mix for Probes <3 Mb, 8 rows of wells, fast hybridization

	Target size <3.0 Mb						
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	7.0 µL	89 µL	149 µL	208 µL	268 µL	402 µL	818 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	15 µL	19 µL	29 µL	58 µL
SureSelect Fast Hybridization Buffer (bottle)	6.0 µL	77 µL	128 µL	179 µL	230 µL	344 µL	701 µL
Probe (with design <3.0 Mb)	2.0 µL	26 µL	43 µL	60 µL	77 µL	115 µL	234 µL
Total Volume	**15.5 µL**	**198.4 µL**	**330.6 µL**	**462 µL**	**593 µL**	**890 µL**	**1811 µL**

Table 71 Preparation of Probe Hybridization master mix for Probes ≥3 Mb, 8 rows of wells, fast hybridization

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4 µL	51 µL	85 µL	119 µL	153 µL	230 µL	468 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	15 µL	19 µL	29 µL	58 µL
SureSelect Fast Hybridization Buffer (bottle)	6 µL	77 µL	128 µL	179 µL	230 µL	344 µL	701 µL
Probe (with design ≥3.0 Mb)	5 µL	64 µL	106 µL	149 µL	191 µL	287 µL	584 µL
Total Volume	**15.5 µL**	**198.4 µL**	**329.6 µL**	**462 µL**	**593 µL**	**890 µL**	**1811 µL**

Table 72 Preparation of Probe Hybridization master mix for Probes <3 Mb, single row of wells, fast hybridization

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	7 µL	11 µL	18 µL	25 µL	32 µL	49 µL	98 µL
RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.3 µL	3.5 µL	7 µL
SureSelect Fast Hybridization Buffer (bottle)	6 µL	9 µL	15 µL	21 µL	27 µL	42 µL	84 µL
Probe (with design <3 Mb)	2 µL	3 µL	5 µL	7 µL	9 µL	14 µL	28 µL
Total Volume	**15.5 µL**	**23.8 µL**	**39.3 µL**	**54.8 µL**	**70.3 µL**	**108.5 µL**	**217 µL**

Table 73 Preparation of Probe Hybridization master mix for Probes ≥3 Mb, single row of wells, fast hybridization

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4 µL	6 µL	10 µL	14 µL	18 µL	28 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 µL
SureSelect Fast Hybridization Buffer (bottle)	6 µL	9 µL	15 µL	21 µL	27 µL	42 µL	84 µL
Probe (with design ≥3 Mb)	5 µL	8 µL	13 µL	18 µL	23 µL	35 µL	70 µL
Total Volume	**15.5 µL**	**23.8 µL**	**38.3 µL**	**54.8 µL**	**70.3 µL**	**108.5 µL**	**217 µL**

Prepare the master mix source plate

10 Using an **Agilent Deep Well** master mix source plate, prepare the hybridization master mix source plate at room temperature, containing the master mixes prepared in **step 8** and **step 9**. Add the volumes indicated in **Table 74** 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 14**.

Table 74 Preparation of the master mix source plate for Hyb_XT_HS2_ILM protocol

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Block master mix	Column 1 (A1-H1)	11 µL	19 µL	27 µL	35 µL	51 µL	103 µL
Probe Hybridization master mix	Column 2 (A2-H2)	23 µL	39 µL	54 µL	70 µL	109 µL	217 µL

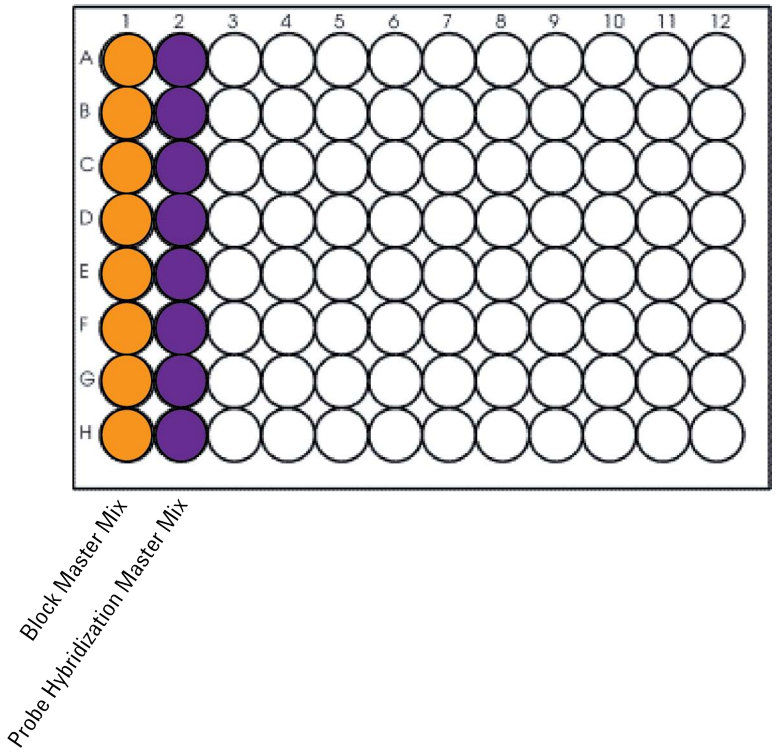


Figure 14 Configuration of the **Agilent Deep Well** master mix source plate for protocol Hyb_XT_HS2_ILM. Column 2 can contain different Probe Hybridization master mixes in each row.

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

Load the Bravo deck

12 Load the Bravo deck according to **Table 75**.

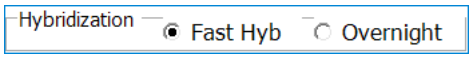
Table 75 Initial Bravo deck configuration for Hyb_XT_HS2_ILM protocol

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

Run VWorks protocol Hyb_XT_HS2_ILM

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

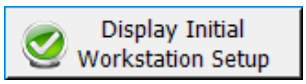
14 Select the **Fast hyb** option.



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

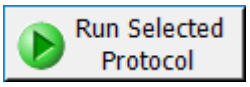
16 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

17 Click **Display Initial Workstation Setup**.



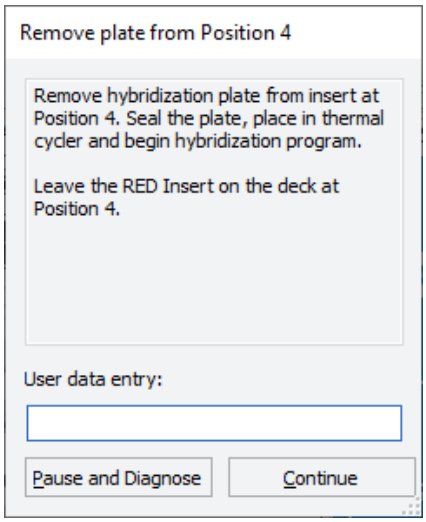
18 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



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

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



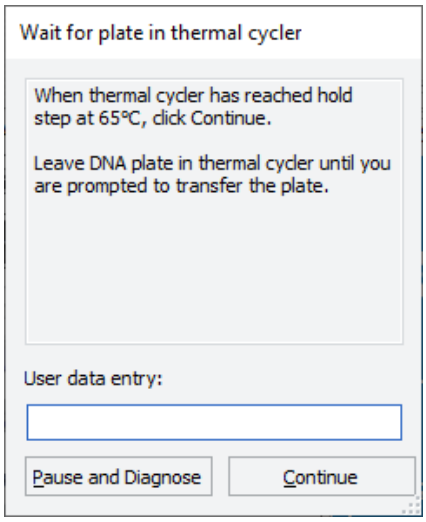
21 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

22 Transfer the sealed plate to a thermal cycler. Initiate the pre-programmed thermal cycler program (**Table 66** on page 96 or **Table 67** on page 96).

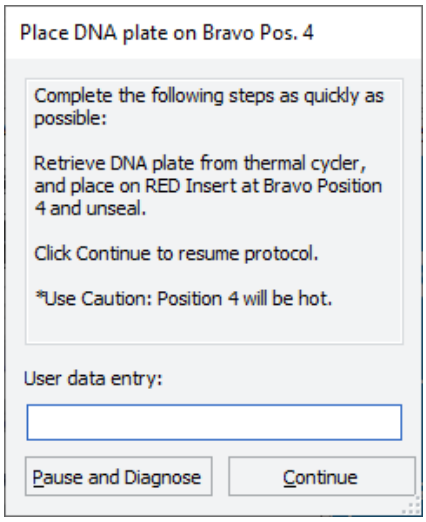
While the sample plate incubates on the thermal cycler, the NGS Workstation aliquots the Probe Hybridization master mix to the Eppendorf twin.tec plate.

CAUTION You must complete **step 23** to **step 27** quickly, and immediately after being prompted by the VWorks software. It is important that sample temperature remains approximately 65°C during transfers between the NGS Workstation and thermal cycler.

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



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

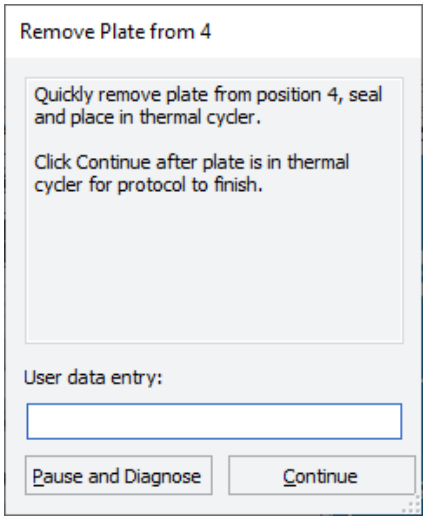


WARNING **Bravo deck position 4 will be hot.**

Use caution when handling components that contact heated deck positions.

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

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



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

27 Quickly transfer the plate back to the thermal cycler, held at 65°C. On the thermal cycler, initiate the hybridization segment of the pre-programmed thermal cycler program (segment 4 from **Table 66** on page 96 or **Table 67** on page 96). During this step, the prepared DNA samples or DNA sample pools are hybridized to the Probe.

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

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

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

30 When the Hybridization protocol is complete, retain the Eppendorf twin.tec source plate containing the Block master mix and Probe Hybridization master mix located at position 6 of the Bravo deck for later use in the Post-CapPCR_XT_HS2_ILM protocol. If you are using the pre-capture pooling workflow, remove the Eppendorf twin.tec plate containing the remainder of the prepared library pools. This plate is located at position 9 of the Bravo deck. Seal the plate and store it at –20°C in the event that the samples require further processing.

31 Continue to **Chapter 7**, "Capture and Amplification" on **page 129** for instructions on running the Capture & Wash protocol. Complete the setup tasks for that protocol (**step 1** on **page 130** through **step 15** on **page 132**) during the thermal cycler incubation for hybridization.

6 Hybridization (Overnight)

This chapter describes the steps to hybridize the DNA library or library pool to the Probe using the overnight hybridization method. The DNA libraries are hybridized to a SureSelect or ClearSeq Probe using single-plex or multi-plex hybridization in a thermal cycler program in which the hybridization segment is 16 to 24 hours.

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 106.
- 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 109.

NOTE For instructions on the fast hybridization method, which also supports both single-plex and multi-plex hybridization, see **Chapter 5**, "Hybridization (Fast)."

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

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 109.

Aliquot prepped DNA samples for hybridization

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

The hybridization reaction requires 500–2000 ng of prepared DNA in a volume of 4 µL. Use the maximum amount of prepared DNA available within this range. This is especially important for libraries prepared from FFPE input DNA.

NOTE

If any of the prepared DNA libraries do not meet the minimum concentration of 125 ng/µL (i.e., 500 ng in 4 µL), then you need to first prepare a concentrated sample of those libraries to be used in the hybridization reactions.

- First, place 500–2000 ng (maximum amount available within this range) of each lower-concentration DNA library into the wells of a 96-well plate or strip tube. Keep on ice.
- Then, using a vacuum concentrator at ≤ 45°C, dehydrate the samples to volume < 4 µL without completely drying them. Measure the final volume of each sample and then bring the volume to 4 µL with nuclease-free water. Record the new concentration.

Using the DNA concentration for each sample (as determined on **page 73** to **page 80** or as recorded after vacuum concentration) calculate the volume of each sample to be used for hybridization using the formula below:

Volume (µL) = 2000 ng/concentration (ng/µL)

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

1. Create a .csv (comma separated value) file with the headers shown in **Figure 15**. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in .csv format. The file must include rows for all 96 wells of the plate.
2. Enter the information requested in the header for each DNA sample.
   - In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
   - In the SourceWell and DestinationWell fields, enter each well position for the plate. SourceWell and DestinationWell field contents must be identical for a given sample.
   - In the Volume field, enter the volume (in µL) of each DNA sample to be used in the hybridization step (see **page 106** for guidelines). For all empty wells on the plate, delete the corresponding rows in the .csv file.

	A	B	C	D
1	SourceBC	SourceWe	Destinatic	Volume
2	abc	A1	A1	3.35
3	abc	B1	B1	3.28
4	abc	C1	C1	2.75
5	abc	D1	D1	3.8
6	abc	E1	E1	3.62
7	abc	F1	F1	2.95
8	abc	G1	G1	3.13
9	abc	H1	H1	2.5
10	abc	A2	A2	3.4

Figure 15 Sample spreadsheet for 1-column run

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Aliquot Input File Templates\Aliquot_Libraries_Template.csv**.

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

3 Load the .csv file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Aliquot Input File Templates**.

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.

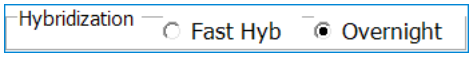
5 Load the Bravo deck according to Table 76.

Table 76 Initial Bravo deck configuration for Aliquot_Libraries protocol

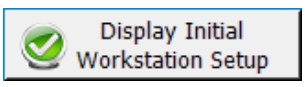
Location	Content
2	Nuclease-free water in Agilent shallow well reservoir (30 mL)
5	Empty Eppendorf twin.tec plate
6	Empty tip box
8	New tip box
9	Prepped library DNA in Eppendorf twin.tec plate

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

7 Select the **Overnight** option.



8 Click **Display Initial Workstation Setup**.



9 Upload the .csv file created in **step 1** through **step 3** on **page 106**.

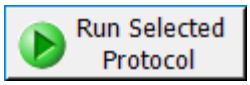
a Click the "**...**" browse button below **Select Aliquot Input File** to open a directory browser window.

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

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

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 5.

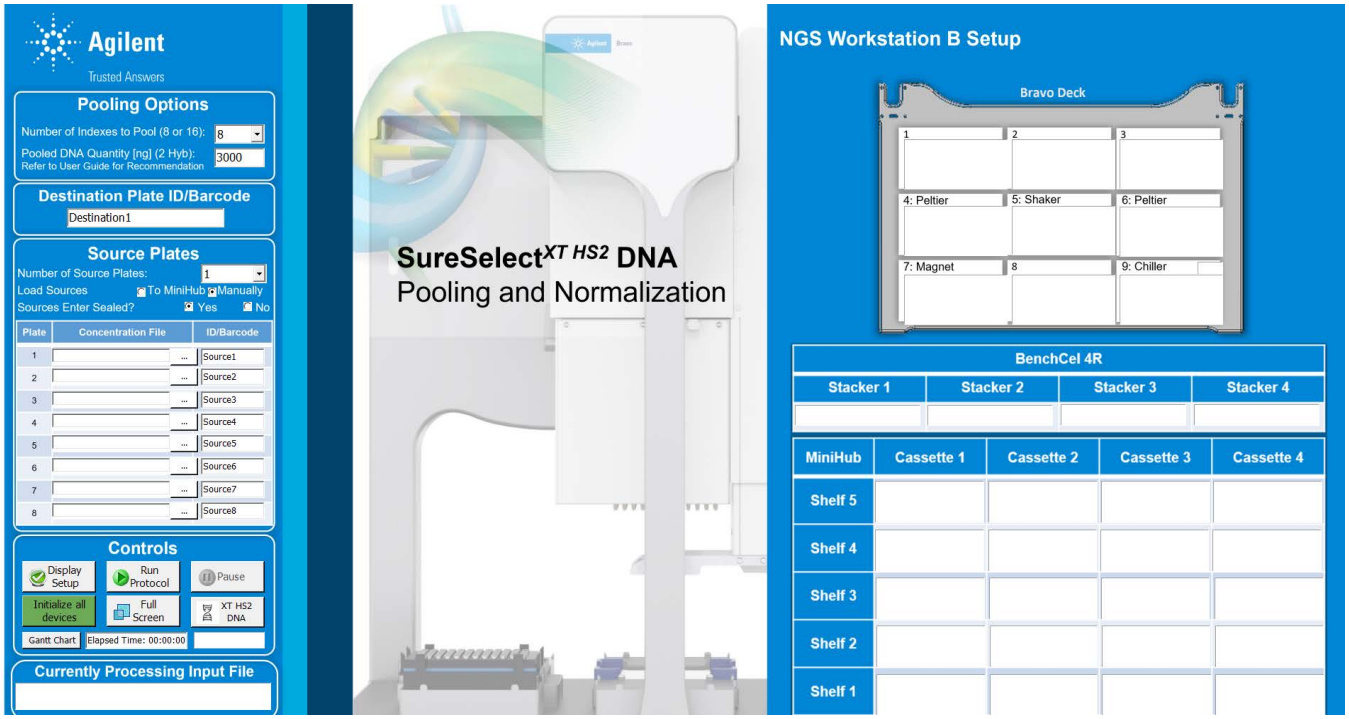
12 Remove the sample plate from the Bravo deck.

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 106.

Pool indexed DNA samples for hybridization

In this step, the NGS Workstation pools the prepped indexed gDNA samples before hybridization to the Probe. This workflow step is set up using the VWorks Form XT_HS2_Pooling.VWForm shown below, which is accessible from within the XT_HS2_ILM form.



Plan pooling run parameters

The hybridization reaction requires 4000 ng of indexed gDNA pool. The pool contains equal amounts of either 8 or 16 individual indexed gDNA libraries. See **Table 77** for the recommended pool composition based on your SureSelect Probe.

Table 77 Pre-capture pooling of indexed DNA libraries

Probe	Amount of total DNA per pool (Amount of DNA pool per hybridization reaction)*	Number of indexed gDNA libraries per pool	Amount of each indexed gDNA library in pool	Maximum DNA concentration for pool
SureSelect XT PreCap Human All Exon and Exome probes	8000 ng (4000 ng/hybridization)	8	500 ng	250 ng/µL
SureSelect XT PreCap Custom Probes	8000 ng (4000 ng/hybridization)	16	250 ng	125 ng/µL

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

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

Accurate normalization of pools requires a minimum pipetting volume of 2 µL for each sample. Maximum DNA concentration values for a pool containing >2 µL of each sample are shown in **Table 77**, above. When higher-concentration DNA samples are included in the pooling run, the DNA pool amount must be adjusted as described below.

- Check the DNA concentration of each sample in the set of source plates to be pooled to a single destination plate to determine the appropriate amount of DNA per pool.
  - If all samples contain DNA at concentrations below the maximum DNA concentration shown in **Table 77**, then prepare pools with 8000 ng of DNA (as shown in the 2nd column in **Table 77**).
  - If at least one of the samples is above the maximum DNA concentration shown in **Table 77**, 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. Continuing with the same example, an Exome probe pool would contain 8 × 400 ng, or 3200 ng DNA.

Plan destination indexed DNA pool sample plate configuration

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

- When using a single Probe for all wells on the plate, fill the plate column-wise in well order A1 to H1, then A2 to H2, ending with A12 to H12.
- When using multiple Probes, configure the plate such that all gDNA library pools to be hybridized to a particular Probe are positioned in appropriate rows. When preparing for the Hyb_XT_HS2_ILM protocol, place samples to be enriched using the same Probe in the same row.
- Each 96-reaction library preparation run produces 6 or 12 gDNA pools. For greatest efficiency of reagent use, gDNA pools from multiple library preparation runs may be placed on the same destination plate for hybridization.

Prepare .csv files for pooling and normalization

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

See **Figure 16** for required .csv file content. Pooling and normalization .csv file templates are provided in the following directory:

C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Pooling and Normalization Templates

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

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

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

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

Figure 16 Sample pooling and normalization .csv file content

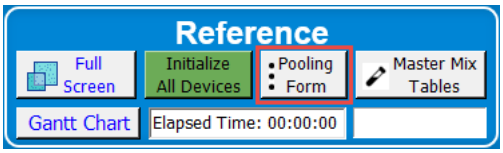
2 In each .csv file, edit the information for each DNA sample (well ID) as follows:
   - In the **PreCap Amplified pond concentrations** field, enter the concentration (in ng/µL) determined on **page 73** for each indexed DNA sample.
   - In the **Target WellID** field, enter the well position of the pool in which the indexed DNA sample should be included for the destination plate. See the guidelines on **page 110** for hybridization sample pool placement considerations.

Set up and run the PreCapture_Pooling automation protocol

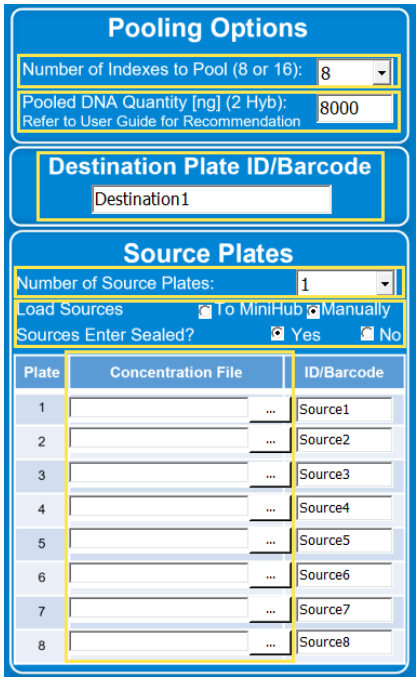
3 Turn on the chiller, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.

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

5 To set up the PreCapture_Pooling automation protocol, open the VWorks Form XT2_HS2_Pooling using one of the methods below.
   - Double-click the shortcut on your desktop for the XT2_HS2_Pooling VWorks Form.
   - In the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Forms** open the file **XT_HS2_Pooling_v.B1.0.VWForm**.
   - From the XT_HS2_ILM VWorks Form, under **Reference**, click **Pooling Form.**



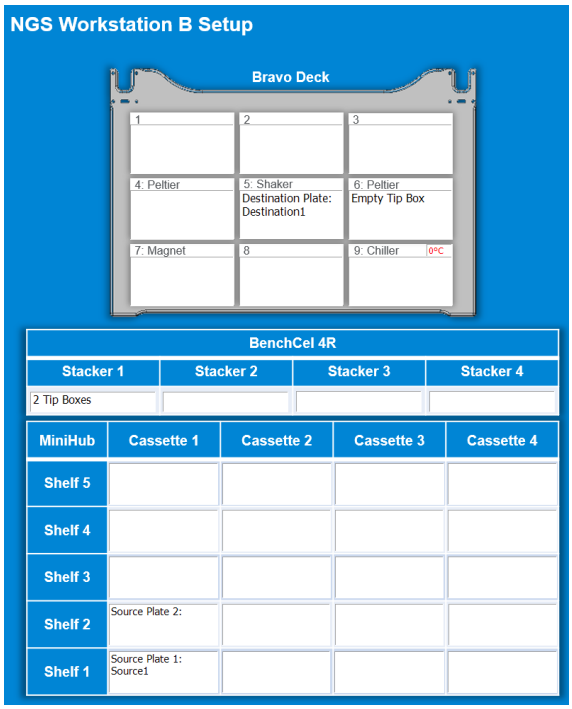
6 In the XT2_HS2_Pooling Form, enter the run information highlighted below:
   - Under **Pooling Options**, from **Number of Indexes to Pool** menu, select 8 or 16 (see **Table 77** on page 110 for guidelines).
   - Under **Pooling Options**, from **Pooled DNA Quantity** menu, enter 8000 ng.
     - 8000 ng is the required amount for all probes when using overnight hybridization (see **Table 77** on page 110). This amount is sufficient for two hybridization reactions.
   - Under **Destination Plate ID/Barcode**, enter the name or barcode of the destination plate into the field provided.
   - Under **Source Plates**, from **Number of Source Plates** menu, select the number of indexed DNA source plates to be provided for sample pooling. If >8 plates will be used to create a single hybridization sample plate, run the pooling and normalization protocol in sets of 8 source plates.
   - Under **Source Plates**, specify whether the indexed DNA source plates will be loaded in the MiniHub and will be sealed at start of run (recommended).
   - In the table under **Source Plates**, in the **Concentration File** field, use the browse button to specify the location of each .csv file that provides sample position and concentration data for each plate.



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

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

- Load each indexed DNA source plates onto its assigned shelf on the MiniHub.
- Load an Eppendorf twin.tec destination plate 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.



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

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

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

10 Remove the destination plate from Bravo deck position 5.

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

Adjust final concentration of pooled DNA

Prior to hybridization with the Probe, use the Aliquot_Water and AMPureXP_XT_HS2_ILM (Concentration of Pool) automation protocols to concentrate each DNA library pool to 8 µL, a volume sufficient for two hybridization reactions. First, the Aliquot_Water protocol adds enough water to each DNA library pool to bring the volume to 100 µL. Then, the AMPureXP_XT_HS2_ILM protocol uses AMPure XP beads to purify the DNA library pools, eluting the DNA in a volume of 8 µL.

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

Prepare .csv file for normalizing sample volumes to 100 µL

1 Create a .csv (comma separated value) file with the headers shown in **Figure 17**. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in .csv format. The file must include rows for all 96 wells of the plate.

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

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

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

Figure 17 Sample Aliquot_Water .csv file content

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Aliquot Input File Templates**.

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

3 Load the .csv file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\XT_HS2_ILM_v.B1.1.2\Pooling and Normalization Templates**.

Set up and run the Aliquot_Water automation protocol

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

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

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

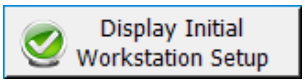
6 Load the Bravo deck according to **Table 78**.

Table 78 Initial Bravo deck configuration for Aliquot_Water protocol

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

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

8 Click **Display Initial Workstation Setup**.



9 Upload the .csv file created in **step 1** through **step 3** on **page 114**.

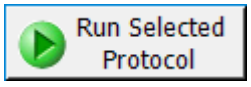
   a Click the "**...**" browse button below **Select Aliquot Input File** to open a directory browser window.

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

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

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

12 Remove the sample plate from the Bravo deck.

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

Set up and run the AMPureXP_XT_HS2_ILM (Concentration of Pool) automation protocol

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

15 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.

16 Pre-set the temperature of Bravo deck position 4 to 45°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.

17 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.

18 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.

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

20 Load the Labware MiniHub according to **Table 79**, using the plate orientations shown in **Figure 4** on page 53.

Table 79 Initial MiniHub configuration for AMPureXP_XT_HS2_ILM (Concentration of Pool) protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Aliquotted AMPure XP beads in Agilent deep well plate from **page 42** (180 µL of beads/well)	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	Empty Eppendorf twin.tec Plate	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 18**		—
Shelf 1 (Bottom)	—	70% ethanol reservoir from **step 19**	—	Empty tip box

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

Table 80 Initial Bravo deck configuration for AMPureXP_XT_HS2_ILM (Concentration of Pool) protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
9	Eppendorf twin.tec plate containing DNA library pools from the Aliquot_Water protocol seated in red insert

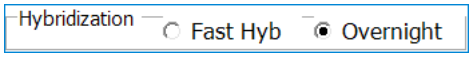
22 Load the BenchCel Microplate Handling Workstation according to **Table 81**.

Table 81 Initial BenchCel configuration for AMPureXP_XT_HS2_ILM (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	—	—	—

23 On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_XT_HS2_ILM (Concentration of Pool)** protocol.

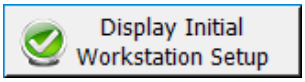
24 Select the **Overnight** option..



25 Under **Select labware for thermal cycling**, select **96 Eppendorf Twin.tec PCR in Red Alum Insert**.

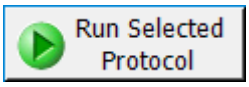
26 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

27 Click **Display Initial Workstation Setup**.



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

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



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

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

In this step, automation protocol Hyb_XT_HS2_ILM is used to complete the liquid handling steps to set up the hybridization reactions. Afterward, you transfer the sample plate to a thermal cycler, held at 65°C, to allow hybridization of the DNA samples to the probe.

This step uses the components listed in **Table 82**. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 82 Reagents for Hybridization

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect XT HS2 Blocker Mix (blue cap)	SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 2 (Post PCR), stored at –20°C	Thaw on ice	**page 121**
SureSelect RNase Block (purple cap)		Thaw on ice	**page 121**
SureSelect Hyb 3 (yellow cap)		Thaw and keep at Room Temperature	**page 119**
SureSelect Hyb 1 (orange cap)	SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 1 (Post PCR), stored at room temperature	Keep at RT, vortex to mix	**page 119**
SureSelect Hyb 2 (red cap)		Keep at RT, vortex to mix	**page 119**
SureSelect Hyb 4 (black cap)		Keep at RT, vortex to mix	**page 119**
Probe	–80°C	Thaw on ice	**page 119**

Program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with either the program in **Table 83** or the program in **Table 84**, depending on the Probe. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

- **Table 83** – Use this program if you are using one of the SureSelect XT HS Human All Exon V8 Probes (V8, V8+UTR, or V8+NCV) or the SureSelect XT HS CRE V4 Probe. These Probes require a longer hybridization on the thermal cycler.
- **Table 84** – Use this program if you are NOT using one of the Probes mentioned above.

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

Table 83 Overnight hybridization program for the *SureSelect XT HS Human All Exon V8 Probes* and *SureSelect XT HS CRE V4 Probe**

Segment	Purpose	Number of Cycles	Temperature†	Time
1	Denaturation	1	95°C	5 minutes
2	Blocking	1	67.5°C	5 minutes
3	Hold for NGS Workstation steps	1	67.5°C	Hold‡
4	Hybridization	1	67.5°C	16–24 hours
5	Hold until start of Capture**	1	67.5°C	Hold‡

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

† If performing the blocking and hybridization steps at 67.5°C does not produce optimal results, reducing the temperature in Segment 2 through Segment 5 from to 67.5°C to 66°C may improve performance.

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

** Samples are held at 67.5°C until they are processed in the Capture & Wash automation protocol that begins on **page 130**.

Table 84 Overnight hybridization program for *all other probes**

Segment	Purpose	Number of Cycles	Temperature	Time
1	Denaturation	1	95°C	5 minutes
2	Blocking	1	65°C	5 minutes
3	Hold for NGS Workstation steps†	1	65°C	Hold
4	Hybridization	1	65°C	16–24 hours
5	Hold until start of Capture‡	1	65°C†	Hold

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

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

‡ Samples are held at 65°C until they are processed in the Capture & Wash automation protocol that begins on **page 130**.

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

Prepare the NGS Workstation

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

3 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.

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 Place red PCR plate inserts at Bravo deck positions 4 and 6.

6 Place an empty tip box on shelf 1 of cassette 4 of the MiniHub.

7 Load tip boxes for the run in the BenchCel Microplate Handling Workstation according to **Table 85**.

Table 85 Initial BenchCel configuration for Hyb_XT_HS2_ILM protocol

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

Prepare the Block master mix and Hybridization Buffer

8 Prepare the appropriate volume of Block master mix, on ice, as indicated in **Table 86**. Mix by vortexing at medium speed for 15–20 seconds. Keep on ice.

Table 86 Preparation of Block master mix for overnight hybridization

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	32 µL	53 µL	74 µL	96 µL	138 µL	276 µL
SureSelect XT HS2 Blocker Mix (blue cap)	5 µL	64 µL	106 µL	149 µL	191 µL	276 µL	553 µL
Total Volume	**7.5 µL**	**96 µL**	**159 µL**	**223 µL**	**287 µL**	**414 µL**	**829 µL**

9 Prepare the appropriate volume of Overnight Hybridization Buffer, at room temperature, as indicated in **Table 87**. Mix by vortexing at medium speed for 15–20 seconds.

If a precipitate forms, warm the Overnight Hybridization Buffer at 65°C for 5 minutes.

Keep the prepared Overnight Hybridization Buffer at room temperature until it is used in **step 10**.

Table 87 Preparation of Overnight Hybridization Buffer

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Hyb 1 (bottle)	6.63 µL	90 µL	149 µL	209 µL	269 µL	403 µL	820 µL
SureSelect Hyb 2 (red cap)	0.27 µL	3.6 µL	6.1 µL	8.5 µL	10.9 µL	16.4 µL	33 µL
SureSelect Hyb 3 (yellow cap)	2.65 µL	36 µL	60 µL	83 µL	107 µL	161 µL	328 µL
SureSelect Hyb 4 (black cap)	3.45 µL	47 µL	78 µL	109 µL	140 µL	210 µL	427 µL
Total Volume	**13.0 µL**	**176.6 µL**	**293.1 µL**	**409.5 µL**	**526.9 µL**	**790.4 µL**	**1608 µL**

Prepare one or more Probe Hybridization master mixes

10 Prepare the appropriate volume of Probe Hybridization master mix for each of the Probes that will be used for hybridization as indicated in **Table 88** to **Table 91**. 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 88** or **Table 89**) on **page 122**.

For runs that use different Probes for individual rows, prepare each master mix as described in Step b (**Table 90** or **Table 91**) on **page 123**.

a For runs that use a single Probe for all rows, prepare a master mix as described in **Table 88** or **Table 89**, according to the probe design size.

Table 88 Preparation of Probe Hybridization master mix for Probes <3 Mb, 8 rows of wells, overnight-hyb

	Target size <3.0 Mb						
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	57 µL	96 µL	134 µL	172 µL	258 µL	526 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	15 µL	19 µL	29 µL	58 µL
Overnight Hybridization Buffer (prepared in **step 9**)	13 µL	166 µL	276 µL	387 µL	497 µL	746 µL	1519 µL
Probe (with design <3.0 Mb)	2 µL	26 µL	43 µL	60 µL	77 µL	115 µL	234 µL
Total Volume	**20 µL**	**255.4 µL**	**425.6 µL**	**596 µL**	**765 µL**	**1148 µL**	**2337 µL**

Table 89 Preparation of Probe Hybridization master mix for Probes ≥3 Mb, 8 rows of wells, overnight-hyb

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	19 µL	32 µL	45 µL	57 µL	86 µL	175 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	15 µL	19 µL	29 µL	58 µL
Overnight Hybridization Buffer (prepared in **step 9**)	13 µL	166 µL	276 µL	387 µL	497 µL	746 µL	1519 µL
Probe (with design ≥3.0 Mb)	5 µL	64 µL	106 µL	149 µL	191 µL	287 µL	584 µL
Total Volume	**20 µL**	**255.4 µL**	**424.6 µL**	**496 µL**	**764 µL**	**1148 µL**	**2336 µL**

b For runs that use different Probes in individual rows, prepare a master mix for each Probe as listed in **Table 90** or **Table 91**, according to the probe design size. The volumes listed in **Table 90** and **Table 91** are for a single row of sample wells. If a given Probe will be hybridized in multiple rows, multiply each of the values below by the number of rows assigned to that Probe.

Table 90 Preparation of Probe Hybridization master mix for Probes <3 Mb, single row of wells, overnight-hyb

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	6.8 µL	11.3 µL	15.8 µL	20 µL	31 µL	63 µL
RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.3 µL	3.4 µL	7 µL
Overnight Hybridization Buffer (prepared in **step 9**)	13 µL	20 µL	33 µL	46 µL	59 µL	90 µL	183 µL
Probe (with design <3 Mb)	2 µL	3 µL	5 µL	7 µL	9 µL	14 µL	28 µL
Total Volume	**20 µL**	**30.6 µL**	**50.6 µL**	**70.6 µL**	**90.3 µL**	**138.4 µL**	**281 µL**

Table 91 Preparation of Probe Hybridization master mix for Probes ≥3 Mb, single row of wells, overnight-hyb

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	2.3 µL	3.8 µL	5.3 µL	7 µL	10 µL	21 µL
RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.3 µL	3.4 µL	7 µL
Overnight Hybridization Buffer (prepared in **step 9**)	13 µL	20 µL	33 µL	46 µL	59 µL	90 µL	183 µL
Probe (with design ≥3 Mb)	5 µL	8 µL	13 µL	18 µL	23 µL	34 µL	70 µL
Total Volume	**20 µL**	**31.1 µL**	**51.1 µL**	**71.1 µL**	**91.3 µL**	**137.4 µL**	**281 µL**

Prepare the master mix source plate

11 Using an **Agilent Deep Well** master mix source plate, prepare the hybridization master mix source plate at room temperature, containing the master mixes prepared in **step 8** and **step 10**. Add the volumes indicated in **Table 92** 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 92 Preparation of the master mix source plate for Hyb_XT_HS2_ILM protocol

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Block master mix	Column 1 (A1-H1)	11 µL	19 µL	27 µL	35 µL	51 µL	103 µL
Probe Hybridization master mix	Column 2 (A2-H2)	30 µL	50 µL	70 µL	90 µL	138 µL	281 µL

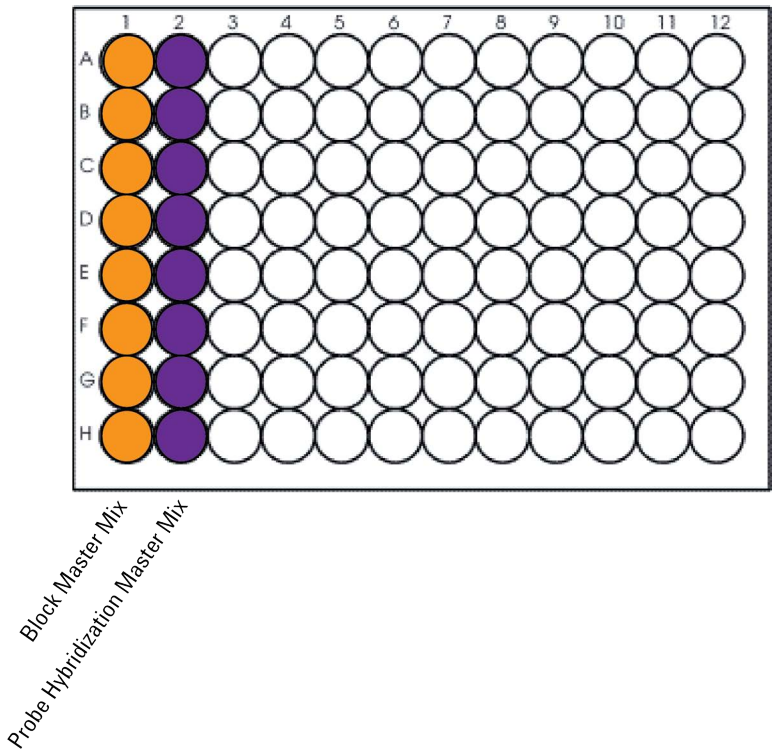


Figure 18 Configuration of the **Agilent Deep Well** master mix source plate for protocol Hyb_XT_HS2_ILM. Column 2 can contain different Probe Hybridization master mixes in each row.

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

Load the Bravo deck

13 Load the Bravo deck according to **Table 93**.

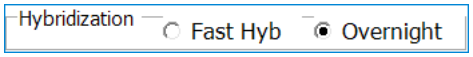
Table 93 Initial Bravo deck configuration for Hyb_XT_HS2_ILM protocol

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

Run VWorks protocol Hyb_XT_HS2_ILM

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

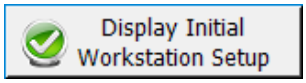
15 Select the **Overnight** option.



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

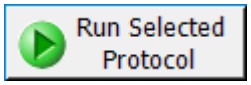
17 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

18 Click **Display Initial Workstation Setup**.



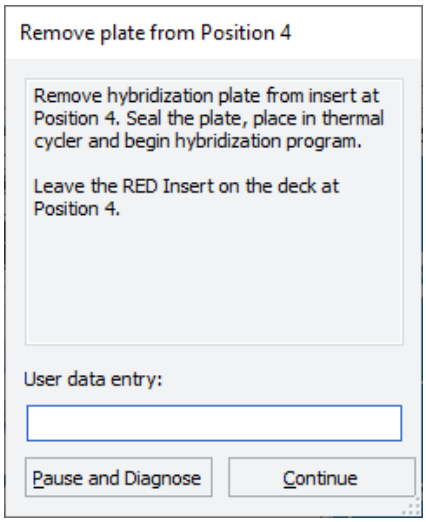
19 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



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

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



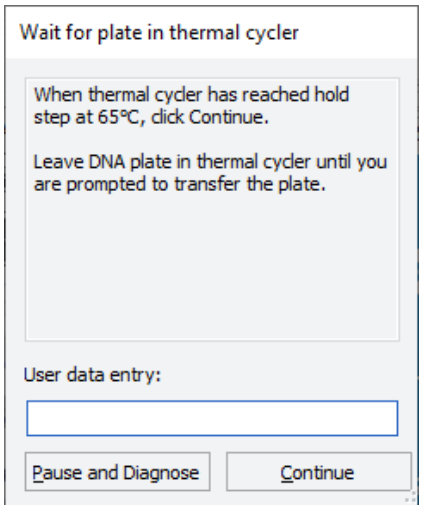
22 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

23 Transfer the sealed plate to a thermal cycler. Initiate the pre-programmed thermal cycler program (**Table 83** on page 120).

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

CAUTION You must complete **step 24** to **step 28** quickly, and immediately after being prompted by the VWorks software. It is important that sample temperature remains approximately 65°C during transfers between the NGS Workstation and thermal cycler.

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



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

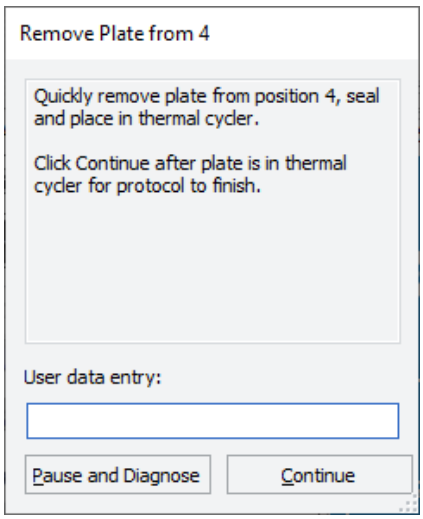
WARNING

Bravo deck position 4 will be hot.

Use caution when handling components that contact heated deck positions.

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

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



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

28 Quickly transfer the plate back to the thermal cycler, held at 65°C. On the thermal cycler, initiate the hybridization segment of the pre-programmed thermal cycler program (segment 4 from **Table 83** on page 120). During this step, the prepared DNA samples or DNA sample pools are hybridized to the Probe.

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

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

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

31 If you are using the pre-capture pooling workflow, when the Hybridization protocol is complete, remove the Eppendorf twin.tec plate containing the remainder of the prepared library pools. This plate is located at position 9 of the Bravo deck. Seal the plate and store it at –20°C in the event that the samples require further processing.

32 After the thermal cycler program completes the next day, continue to **Chapter 7**, "Capture and Amplification" on **page 129** for instructions on running the Capture & Wash protocol.

7 Capture and Amplification

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

Step 1. Capture and wash the hybridized gDNA

This step uses runset SSELCapture&Wash_XT_HS2_ILM to automate capture of the gDNA-probe hybrids using streptavidin-coated magnetic beads.

NOTE

For the fast hybridization workflow, setup tasks for the Capture & Wash protocol (**step 1**, below, through **step 15** on **page 132**) should be completed during the thermal cycler incubation for hybridization (approximately 1–2 hour duration) started on **page 104**.

The Capture & Wash runset uses the components listed in **Table 94**.

Table 94 Reagents for Capture

Kit Component	Storage Location	Where Used
SureSelect Binding Buffer	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	**page 131**
SureSelect Wash Buffer 1	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	**page 131**
SureSelect Wash Buffer 2	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	**page 131**
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 3** on page 13)	**page 131**

Prepare the NGS Workstation

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

Prepare the Streptavidin beads

5. Vigorously resuspend the Streptavidin T1 magnetic beads on a vortex mixer. The beads settle during storage.
6. Wash the magnetic beads.
   a. In a conical vial, combine the components listed in **Table 95**. The volumes below include the required overage.

Table 95 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.)

7 Resuspend the beads in SureSelect Binding buffer, according to **Table 96** below.

Table 96 Preparation of magnetic beads for SSELCapture&Wash_XT_HS2_ILM 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

8 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.

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

Prepare capture and wash solution source plates

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

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

At the end of the automation protocol, retain this reservoir for use in the Post-CapPCR_XT_HS2_ILM and AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocols.

11 Prepare an Eppendorf twin.tec source plate labeled *Wash #1*. For each well to be processed, add 150 µL of SureSelect Wash Buffer 1.

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

Load the NGS Workstation

13 Load the Labware MiniHub according to **Table 97**, using the plate orientations shown in **Figure 4** on page 53.

Table 97 Initial MiniHub configuration for SSELCapture&Wash_XT_HS2_ILM runset

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	*Wash #1* Eppendorf twin.tec source plate	—	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 10**	—	—
Shelf 1 (Bottom)	—	—	—	Empty tip box

14 Load the Bravo deck according to **Table 98** (position 5 should already be loaded).

Table 98 Initial Bravo deck configuration for SSELCapture&Wash_XT_HS2_ILM runset

Location	Content
1	Empty waste reservoir (Agilent 2 mL square well)
4	Empty red insert
5	Dynabeads streptavidin bead Deep Well source plate
6	*Wash #2* Deep Well source plate seated on silver Deep Well insert

15 Load the BenchCel Microplate Handling Workstation according to **Table 99**.

Table 99 Initial BenchCel configuration for SSELCapture&Wash_XT_HS2_ILM runset

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

Run VWorks runset SSELCapture&Wash_XT_HS2_ILM

Start the SSELCapture&Wash_XT_HS2_ILM runset upon completion of the hybridization incubation. The hybridization incubation is complete when the thermal cycler program reaches the 65°C Hold step.

The total duration of the SSELCapture&Wash_XT_HS2_ILM runset is approximately 2 hours. An operator must be present to complete two actions during the runset at the time points in the table below. The times provided are approximate; each action is completed in response to a VWorks prompt at the appropriate time in the runset.

Table 100Operator actions during the SSELCapture&Wash_XT_HS2_ILM runset

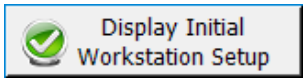
Operator action	Approximate time after run start
Transfer hybridization reactions from thermal cycler to NGS Workstation	<5 minutes
Remove PCR plate from red aluminum insert	<5 minutes

16 On the SureSelect setup form, under **Select protocol to execute**, select the **SSELCapture&Wash_XT_HS2_ILM** runset.

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

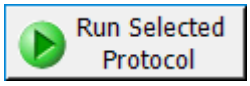
18 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

19 Click **Display Initial Workstation Setup**.

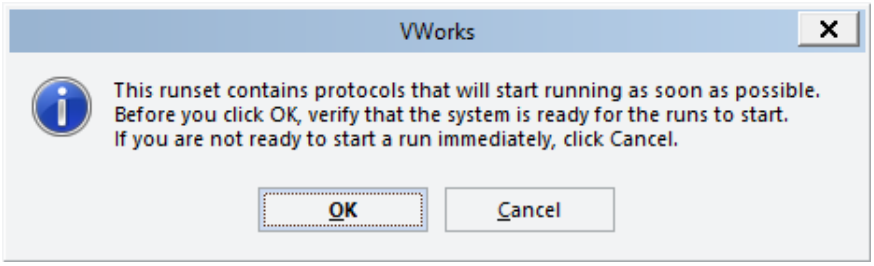


20 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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

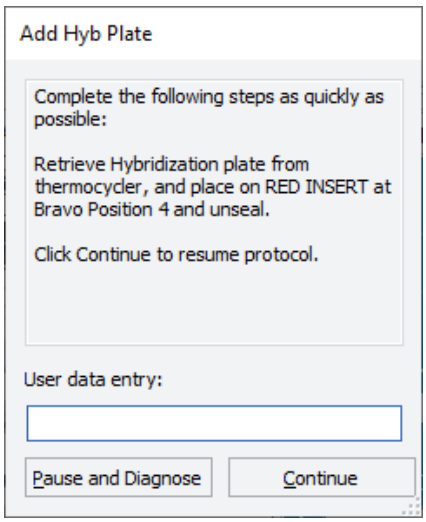


22 When ready to begin the run, click **OK** in the following window.

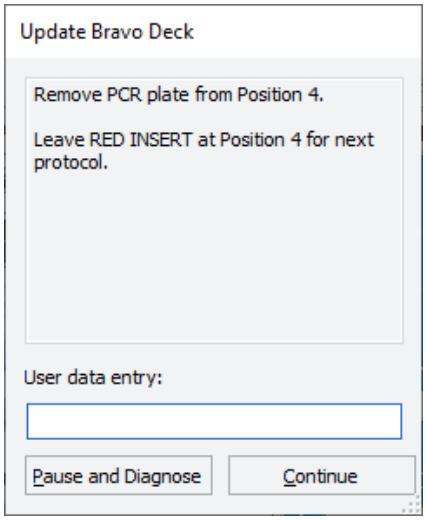


CAUTION It is important to complete **step 23** quickly and carefully. Unseal the plate without tilting or jerking the plate to avoid sample splashing. Make sure that the NGS Workstation is completely prepared, with all components in place, before you transfer the sample plate to the Bravo deck.

23 When prompted by VWorks as shown below, quickly remove the PCR plate, containing the hybridization reactions held at 65°C, from the thermal cycler. Unseal the plate carefully to avoid splashing, and quickly transfer the plate to position 4 of the Bravo deck, seated in the red insert. Click **Continue** to resume the runset.



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



The remainder of the SSELCapture&Wash_XT_HS2_ILM runset takes approximately 2 hours. Once the runset is complete, the captured, bead-bound DNA samples are located in the Eppendorf plate at position 9 of the Bravo deck.

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

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

Step 2. Amplify the captured libraries

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

The design size of your Probe determines the amplification cycle number. Plan your experiments for amplification of samples prepared using probes of similar design sizes on the same plate. See **Table 103** on page 136 for cycle number recommendations.

This step uses the components listed in **Table 101**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 101 Reagents for post-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Pipette up and down 15–20 times	**page 137**
5× Herculase II Reaction Buffer with dNTPs (clear cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Vortexing	**page 137**
SureSelect Post-Capture Primer Mix (clear cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Vortexing	**page 137**

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

Prepare the NGS Workstation

1. Clear the Labware MiniHub and BenchCel of plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water.

   Use the same Agilent shallow well reservoir that was used in the SSELCapture&Wash_XT_HS2 protocol.

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

   At the end of the automation protocol, retain this reservoir for use in the AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocol.

5 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**. Bravo deck positions 4 and 6 correspond to CPAC 2, positions 1 and 2, on the Multi TEC control touchscreen.

Pre-program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 102**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

Table 102 Post-capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10 to 16 See **Table 103** for recommendations based on probe design size	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Table 103 Post-capture PCR cycle number recommendations

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

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

1 Prepare the appropriate volume of post-capture PCR Master Mix, according to **Table 104**. Mix well using a vortex mixer and keep on ice.

Table 104 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	1105 µL
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	17 µL	26 µL	34 µL	43 µL	57 µL	111 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17 µL	26 µL	34 µL	43 µL	57 µL	111 µL
Total Volume	**12 µL**	**204 µL**	**307 µL**	**408 µL**	**511 µL**	**688 µL**	**1327 µL**

2 Using an **Eppendorf twin.tec** master mix source plate, prepare the master mix source plate by adding the volume of PCR master mix indicated in **Table 105** to all wells of column 1 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 19**

Table 105 Preparation of the master mix source plate for Post-CapPCR_XT_HS2_ILM protocol

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
Post-Capture PCR Master Mix	Column 1 (A1-H1)	23 µL	36 µL	49 µL	62 µL	82 µL	163 µL

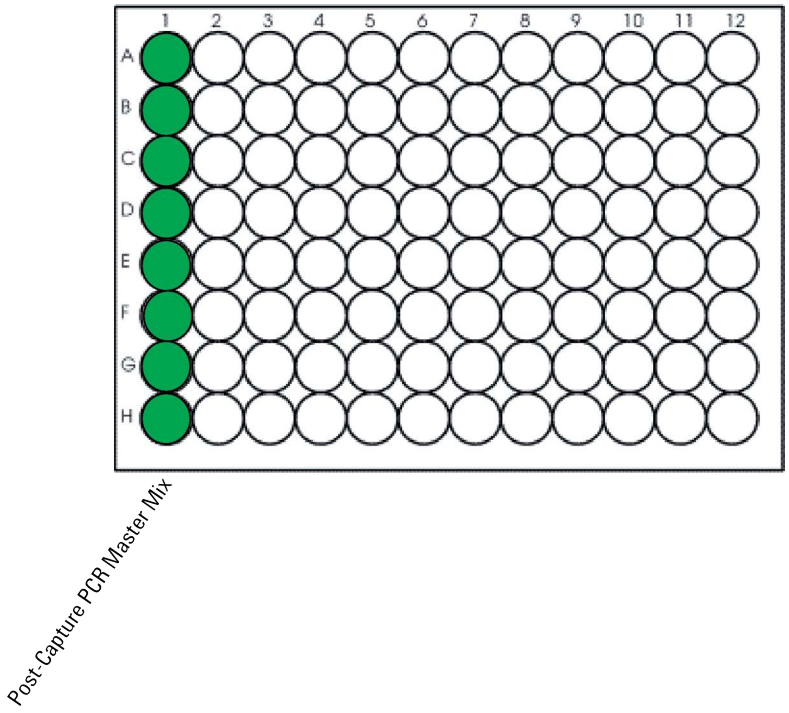


Figure 19 Configuration of the **Eppendorf twin.tec** master mix source plate for protocol Post-CapPCR_XT_HS2_ILM

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

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

Load the NGS Workstation

1 Load the Labware MiniHub according to **Table 106**, using the plate orientations shown in **Figure 4** on page 53.

Table 106 Initial MiniHub configuration for Post-CapPCR_XT_HS2_ILM protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	New tip box	Nuclease-free water reservoir from **step 4**	—	—
Shelf 1 (Bottom)	Empty tip box	—	—	Empty tip box

2 Load the Bravo deck according to **Table 107**.

Table 107 Initial Bravo deck configuration for Post-CapPCR_XT_HS2_ILM protocol

Location	Content
4	Captured DNA bead suspensions in Eppendorf twin.tec plate (unsealed)
6	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
9	Master mix plate containing PCR Master Mix in Column 1 (unsealed)

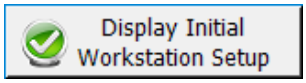
3 Load the BenchCel Microplate Handling Workstation according to **Table 108**.

Table 108 Initial BenchCel configuration for Post-CapPCR_XT_HS2_ILM protocol

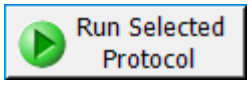
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Run VWorks protocol Post-CapPCR_XT_HS2_ILM

1. On the SureSelect setup form, under S**elect protocol to execute**, select the **Post-CapPCR_XT_HS2_ILM** protocol.
2. Under **Select labware for thermal cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.
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**.

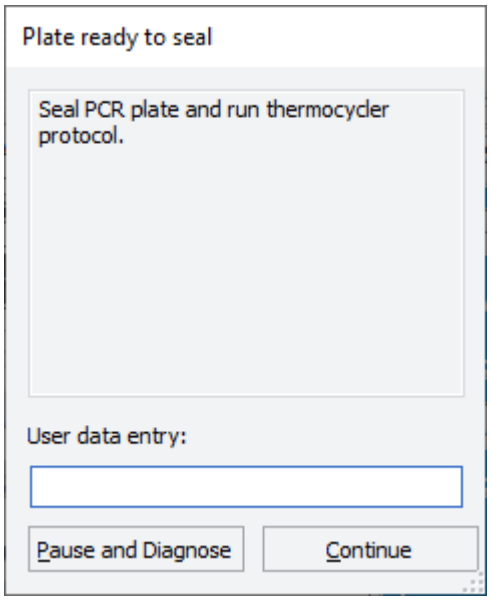


5. Verify that the NGS Workstation has been set up as displayed on the right side of the form.
6. When verification is complete, click **Run Selected Protocol**.



Running the Post-CapPCR_XT_HS2_ILM protocol takes approximately 15 minutes. Once complete, the PCR-ready samples, containing captured DNA and PCR master mix, are located in the PCR plate at position 6 of the Bravo deck.

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



8. Place the plate in the thermal cycler. Resume the thermal cycler program in **Table 102** on page 136.
9. When the PCR amplification program is complete, spin the plate briefly then keep on ice.

Step 3. Purify the amplified indexed libraries using AMPure XP beads

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

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

Prepare the NGS Workstation and reagents

1. Retain the Eppendorf twin.tec source plate containing the Post-Capture PCR master mix located at position 9 of the Bravo deck for later use in the TS_HighSensitivity_D1000 protocol (see **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and High Sensitivity D1000 ScreenTape"** on page 144). Otherwise, clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 45°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
5. Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water.

   Use the same Agilent shallow well reservoir that was used in the Post-CapPCR_XT_HS2_ILM protocol.

   Make sure that the water in the reservoir does not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.
6. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.
7. Load the Labware MiniHub according to **Table 109**, using the plate orientations shown in **Figure 4** on page 53.

Table 109 Initial MiniHub configuration for AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Aliquotted AMPure XP beads in Agilent Deep Well plate from **page 44** (50 µL of beads/well)	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	Empty Eppendorf twin.tec Plate	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 5**		—
Shelf 1 (Bottom)	—	70% ethanol reservoir from **step 6**	—	Empty tip box

8 Load the Bravo deck according to **Table 110**.

Table 110 Initial Bravo deck configuration for AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocol

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

9 Load the BenchCel Microplate Handling Workstation according to **Table 111**.

Table 111 Initial BenchCel configuration for AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocol

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

Run VWorks protocol AMPureXP_XT_HS2_ILM (Post-Capture PCR)

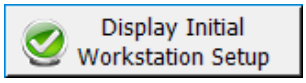
1 On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_XT_HS2_ILM (Post-Capture PCR)** protocol.

NOTE AMPureXP purification protocols are used during multiple steps of the SureSelect automation workflow. Be sure to select the correct workflow step when initiating the automation protocol.

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

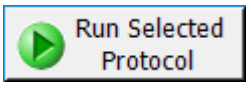
3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

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**.



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

Step 4. Assess sequencing library DNA quantity and quality

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

- Option 1: Prepare the analytical assay plate using automation (protocol TS_HighSensitivity_D1000) and perform analysis on Agilent 4200 TapeStation. See **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and High Sensitivity D1000 ScreenTape"** on page 144.
- 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 149.

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. Clear the Labware MiniHub and BenchCel of plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn off the ThermoCube device (see **page 22**) to restore position 9 of the Bravo deck to room temperature.
4. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
5. Using the same **Eppendorf twin.tec** master mix source plate that was used for the Post-CapPCR_XT_HS2_ILM protocol run, prepare the Sample Buffer source plate at room temperature. Add the volume of High Sensitivity D1000 Sample Buffer indicated in **Table 112** to each well of column 2 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 20**.

Table 112 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 Eppendorf twin.tec 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 2 (A2-H2)	8 µL	11 µL	14 µL	17 µL	23 µL	44 µL

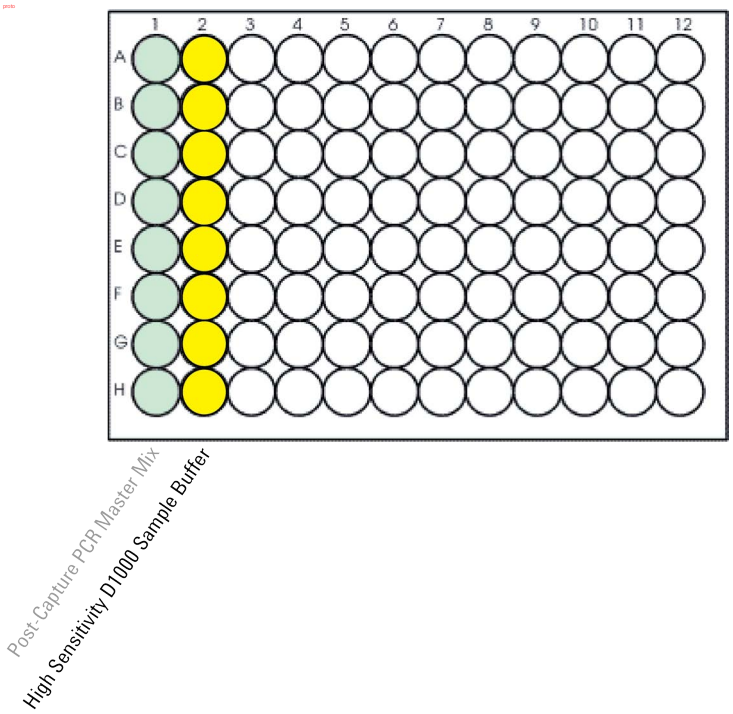


Figure 20 Configuration of the **Eppendorf twin.tec** source plate for protocol TS_High-Sensitivity_D1000. Column 1 was used during a previous protocol.

Load the NGS Workstation

6 Load the Labware MiniHub according to **Table 113**, using the plate orientations shown in **Figure 4** on page 53.

Table 113 Initial MiniHub configuration for TS_HighSensitivity_D1000 protocol

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

7 Load the Bravo deck according to **Table 114**.

Table 114 Initial Bravo deck configuration for TS_HighSensitivity_D1000 protocol

Location	Content
4	Amplified post-capture libraries or library pools in Eppendorf twin.tec plate (unsealed)
6	Empty Eppendorf twin.tec plate
9	Eppendorf twin.tec source plate containing High Sensitivity D1000 Sample Buffer in Column 2

CAUTION

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

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

8 Load the BenchCel Microplate Handling Workstation according to **Table 115**.

Table 115 Initial BenchCel configuration for TS_HighSensitivity_D1000 protocol

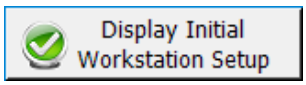
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Run VWorks protocol TS_HighSensitivity_D1000

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

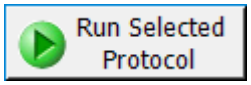
10 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

11 Click **Display Initial Workstation Setup**.



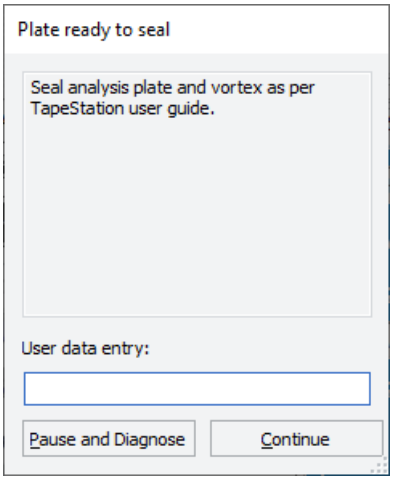
12 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



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 you proceed to the steps on **page 151**.

14 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

15 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.

16 Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 116** for guidelines). Sample electropherograms are shown in **Figure 21** (library prepared from high-quality DNA), **Figure 22** (library prepared from medium-quality FFPE DNA), and **Figure 23** (library prepared from low-quality FFPE DNA).

Table 116 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

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

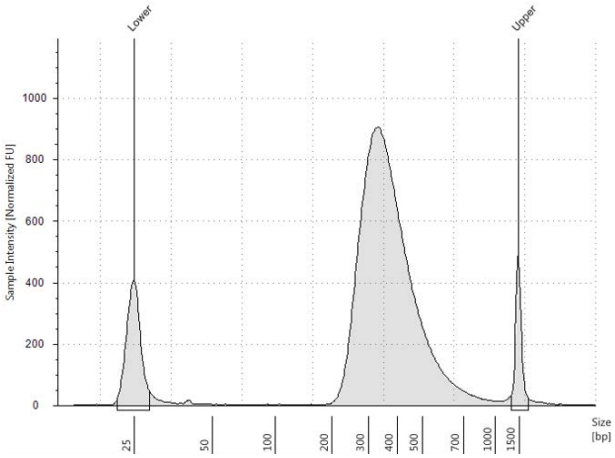


Figure 21 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

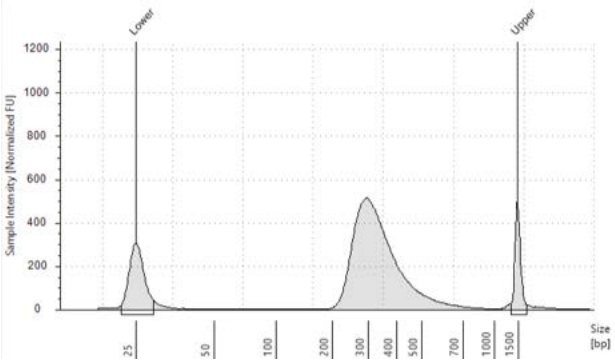


Figure 22 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

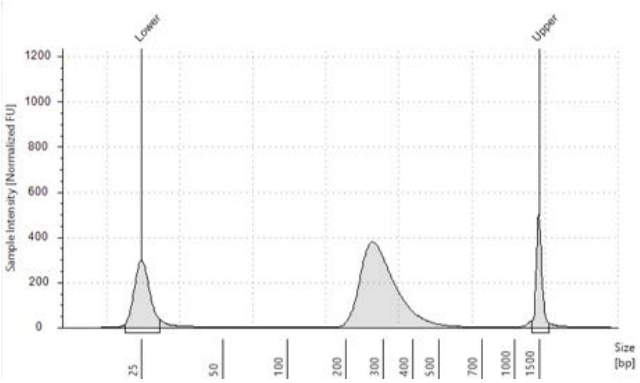


Figure 23 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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 21** through **Figure 23**). Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 116** for guidelines). **Table 117** includes links to assay instructions.

Table 117 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.

8 NGS and Analysis Guidelines

This chapter provides sample pooling instructions for the post-capture pooling workflow as well as instructions and resources for the sequencing and analysis steps.

Step 1. Optional: Pool samples for multiplexed sequencing

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 the sequencing samples"** on page 156.

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. The formula below is provided for determination of the amount of each indexed sample to add to the pool.

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

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

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

is the number of indexes, and

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

Table 118 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 118 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 24**. 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 24** 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 24 Sample spreadsheets for method 1 and method 2

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Aliquot Input File Templates\Aliquot_Captures_Template.csv**.

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

3 Load the .csv file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.B1.1.2\Aliquot Input File Templates**.

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.

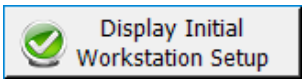
5 Load the Bravo deck according to **Table 119**.

Table 119 Initial Bravo deck configuration for Aliquot_Captures protocol

Location	Content
5	Empty Agilent Deep Well plate
6	Empty tip box
8	New tip box
9	Purified amplified indexed libraries in Eppendorf twin.tec plate

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

7 Click **Display Initial Workstation Setup**.



8 Upload the .csv file created in **step 1** through **step 3** (page **153** to **154**).

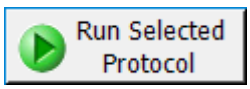
a Click the "**...**" browse button below **Select Aliquot Input File** to open a directory browser window.

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

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

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

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



The aliquoting protocol takes approximately 1 hour for 96 samples. When complete, the destination plate containing the library pools is on Bravo deck position 5.

11 Remove the destination plate from the Bravo deck.

12 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 the sequencing samples

The final SureSelect XT HS2 library pool is ready for 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 Illumina sequencers, as shown in **Figure 25**.

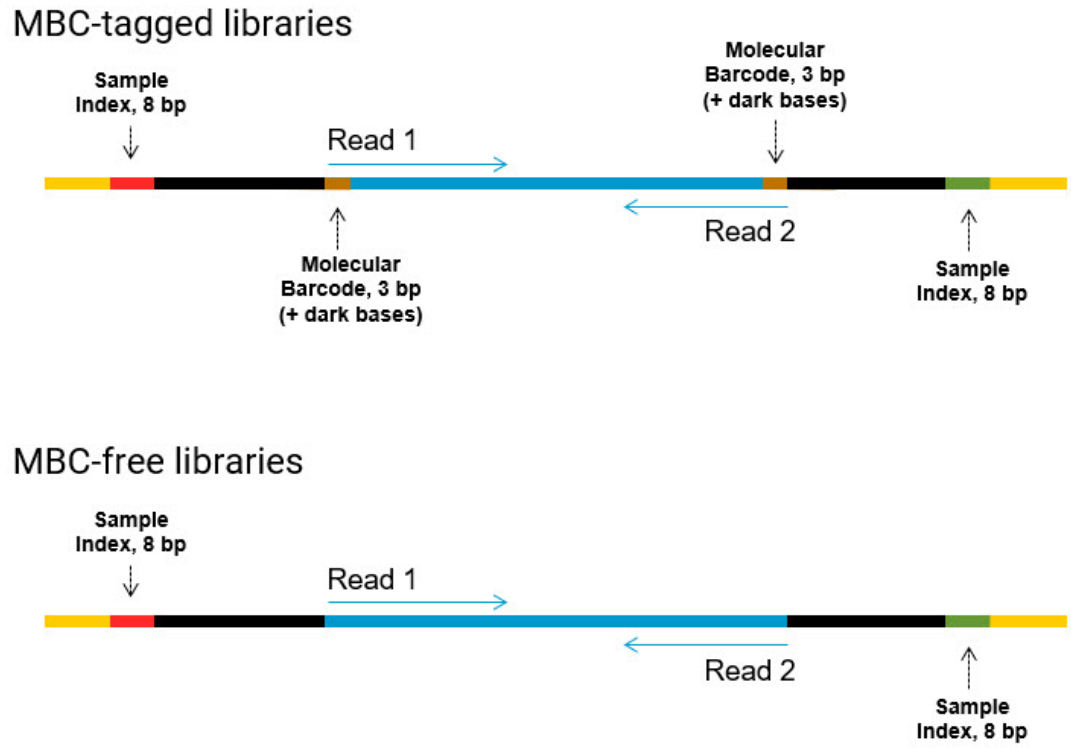


Figure 25 Content of SureSelect XT HS2 sequencing libraries. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the dual sample indexes (red and green), library PCR primers (yellow), and optional molecular barcodes (brown).

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. **Table 120** 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 120 Illumina Kit Configuration Selection Guidelines

Instrument	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
MiSeq	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	9–10 pM
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 4000	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1	300–400 pM
NovaSeq 6000	Standard Workflow Runs	2 × 100 bp or 2 × 150 bp	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 120** 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 121** showing example settings for 2x150 bp sequencing.

Table 121 Run settings for 2x150 bp sequencing

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

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

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

- Each of the sample-level indexes (i7 and i5) requires an 8-bp index read. For complete index sequence information, see **Table 141** on page 174 through **Table 148** on page 181.
- 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 141** through **Table 148** 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 159** 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 160** 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 161**.

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.
- When setting up FASTQ file uploads for each sample, select the **Application Chemistry** menu option required for your adaptor type, as shown in **Table 122**.

Table 122 Alissa Reporter Application Chemistry selection based on Adaptor type

HS2 Library Adaptor Type	Application Chemistry Selection Required
MBC-free	*XTHS2 (no MBC)*
MBC-tagged	*XTHS2*

- 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 123**.

Table 123 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 SureSelect XT HS2 DNA NGS 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.

The type of SureSelect HS2 adaptor used for sample library preparation determines the correct read pre-processing workflow for the sample, as summarized in **Table 124**. Use of the AGeNT read processing tools is outlined briefly below. See the AGeNT Best Practices document sections covering the workflow suitable for your SureSelect libraries for more information.

Table 124 Adaptor-based AGeNT workflow selection

HS2 Library Adaptor Type	Suitable AGeNT Workflow
MBC-free	SureSelect XT
MBC-tagged	SureSelect XT HS2

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

The trimmed reads should be aligned (and MBC tags added to the aligned BAM file where applicable) using a suitable tool such as BWA-MEM.

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 121** on page 157). 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 121** on page 157). 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 25**), which may affect alignment quality.

9 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 125**.

Table 125 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 48**	Qualification of DNA Integrity	Not required	Required
Enzymatic fragmentation duration **page 51**	Duration of the 37°C fragmentation step	15–25 minutes, depending on read length requirements	25 minutes
DNA input for Library Preparation **page 49**	Input amount and means of quantification	10 ng to 200 ng, quantified by Qubit assay	Based on determined DNA integrity (see **Table 20** on page 49 and **Table 21** on page 49)
DNA Shearing **page 58**	Mode of DNA Shearing	2 × 120 seconds	240 seconds (continuous)
Pre-capture PCR **page 66**	Cycle number	8–11	11–14
Sequencing **page 166**	Output augmentation	Per project requirements	1× to 10× based on determined DNA integrity (see **Table 126** and **Table 127** on page 166)

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 126**. 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 126 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 127**. 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 127 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

10 Reference

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

Kit Contents

The SureSelect XT HS2 DNA System protocol using the NGS Workstation uses the kits listed in **Table 128**. Detailed contents of each of the multi-part component kits listed in **Table 128** are shown in **Table 129** through **Table 139** on the following pages.

Table 128 Component Kits

Purchased Kit Name [Kit p/n]	Workflow Restrictions	Component Kit Name [Kit p/n]	Storage Condition
SureSelect XT HS2 DNA Library Preparation Kit (Pre PCR), 96 reactions [G9985A through G9985D]	MBC-tagged libraries only	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)] [5191-5691 (Index Pairs 289–384)]	–20°C
SureSelect XT HS2 DNA Library Preparation Kit with MBC-free adaptors (Pre PCR), 96 reactions [G9956A through G9956D]	MBC-free libraries only	SureSelect XT HS2 Library Preparation Kit for ILM, MBC-Free (Pre PCR) [5282-0052]	–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)] [5191-5691 (Index Pairs 289–384)]	–20°C
SureSelect XT HS2 DNA Reagent Kit, 96 Reactions [G9983A through G9983D; or G9984A through G9984D with AMPure XP/ Streptavidin Beads]	MBC-tagged libraries only Fast hybridization only Post-capture pooling workflow	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)] [5191-5691 (Index Pairs 289–384)]	–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 XT HS2 DNA Target Enrichment Kit (Post PCR), 12 Hybs [G9987A]	Fast hybridization only Pre-capture pooling workflow	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

Table 128 Component Kits (continued)

Purchased Kit Name [Kit p/n]	Workflow Restrictions	Component Kit Name [Kit p/n]	Storage Condition
SureSelect XT HS2 Target Enrichment Kit (Post PCR), Overnight Hybridization, 12 Hybs [G9957A]	Overnight hybridization only Pre-capture pooling workflow	SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 1 (Post PCR) [5282-0049]	Room Temperature
		SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 2 (Post PCR) [5282-0048]	–20°C
		SureSelect Streptavidin Beads [5191-5742]	+4°C
SureSelect XT HS2 Target Enrichment Kit, 96 Hybridizations [G9987B]	Fast hybridization only Post-capture pooling workflow	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 Streptavidin Beads [5191-5742]	+4°C
SureSelect XT HS2 Target Enrichment Kit (Post PCR), Overnight Hybridization, 96 Hybs [G9957B]	Overnight hybridization only Post-capture pooling workflow	SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 1 (Post PCR) [5282-0051]	Room Temperature
		SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module, Box 2 (Post PCR) [5282-0050]	–20°C
		SureSelect Streptavidin Beads [5191-5742]	+4°C
SureSelect Enzymatic Fragmentation Kit, 96 Reactions Automation [5191-6764]			–20°C

Table 129 Component Kit p/n 5500-0147 – 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 130 Component Kit p/n 5500-0052 – SureSelect XT HS2 Library Preparation Kit for ILM, MBC-Free (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 MBC-Free 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 131 Component Kit p/n 5191-5688 through 5191-5691 – 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 132 Component Kit p/n 5190-9687 – 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 133 Component Kit p/n 5191-6688 – 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

Table 134 Component Kit p/n 5191-6689 – 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 135 Component Kit p/n 5191-6690 – 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

Table 136 Component Kit p/n 5282-0049 – SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module Box 1 (Post PCR) Content

Kit Component	Format
SureSelect Hyb 1	tube with orange cap
SureSelect Hyb 2	tube with red cap
SureSelect Hyb 4	tube with black cap
SureSelect Binding Buffer	bottle
SureSelect Wash Buffer 1	bottle
SureSelect Wash Buffer 2	bottle

Table 137 Component Kit p/n 5282-0048 – SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module Box 2 (Post PCR) Content

Kit Component	Format
SureSelect Hyb 3	tube with yellow cap
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

Table 138 Component Kit p/n 5282-0051 – SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module Box 1 (Post PCR) Content

Kit Component	Format
SureSelect Hyb 1	bottle
SureSelect Hyb 2	tube with red cap
SureSelect Hyb 4	tube with black cap
SureSelect Binding Buffer	bottle
SureSelect Wash Buffer 1	bottle
SureSelect Wash Buffer 2	bottle

Table 139 Component Kit p/n 5282-0050 – SureSelect XT HS2 Target Enrichment Kit for ILM, Overnight Hyb Module Box 2 (Post PCR) Content

Kit Component	Format
SureSelect Hyb 3	tube with yellow cap
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 182** through **page 183** 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 141** through **Table 147**. 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 140**. 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 140 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 141 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 142 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 143 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 144 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 145 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 146 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 147 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 148 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 149 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 150 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 151 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 152 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 Kits (with options for MBC, Hyb time, and Pooling) using NGS Workstation protocol.

Enzymatic Fragmentation

Table 153 Fragmentation master mix - used on page 52

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	43 µL	60 µL	77 µL	98 µL	136 µL	254 µL
5X SureSelect Fragmentation Buffer (blue cap)	2 µL	43 µL	60 µL	77 µL	98 µL	136 µL	254 µL
SureSelect Fragmentation Enzyme (green cap)	1 µL	21 µL	30 µL	38 µL	49µL	68 µL	127 µL

Table 154 Master mix source plate for enzymatic fragmentation protocol EnzFrag_XT_LI_ILM - used on page 52

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	Column 1 (A1-H1)	13 µL	18 µL	23 µL	29 µL	40 µL	75 µL

Library Preparation

Table 155 End Repair/dA-Tailing master mix - used on page 60

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 156 Ligation master mix - used on page 61

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 µL	489 µL	684 µL	880 µL	1271 µL	2737 µL
T4 DNA Ligase (blue cap)	2 µL	26 µL	43 µL	60 µL	77 µL	111 µL	238 µL
Total Volume	**25 µL**	**319 µL**	**532 µL**	**744 µL**	**956 µL**	**1381 µL**	**2975 µL**

Table 157 Adaptor Oligo Mix dilution - used on page 61

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	43 µL	64 µL	85 µL	106 µL	144 µL	276 µL
XT HS2 Adaptor Oligo Mix (white cap) -- OR -- SureSelect MBC-Free Adaptor Oligo Mix (black cap)	5 µL	85 µL	128 µL	170 µL	213 µL	287 µL	553 µL
Total Volume	**7.5 µL**	**128 µL**	**191 µL**	**255 µL**	**319 µL**	**431 µL**	**829 µL**

Table 158 Master mix source plate for library preparation runset LibraryPrep_XT_LI_ILM - used on page 61

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
End Repair-dA Tailing master mix	Column 2 (A2-H2)	31 µL	52 µL	73 µL	94 µL	136 µL	280 µL
Ligation master mix	Column 3 (A3-H3)	36 µL	62 µL	88 µL	114 µL	166 µL	360 µL
Adaptor Oligo Mix dilution	Column 4 (A4-H4)	15 µL	23 µL	30 µL	38 µL	53 µL	101 µL

Pre-Capture PCR

Table 159 Pre-Capture PCR master mix - used on page 67

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	340 µL	425 µL	574 µL	1066 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17 µL	26 µL	34 µL	43 µL	57 µL	107 µL
Total Volume	**11 µL**	**187 µL**	**281 µL**	**374 µL**	**468 µL**	**631 µL**	**1173 µL**

Table 160 Master mix source plate for pre-capture PCR protocol Pre-CapPCR_XT_LI_ILM - used on page 67

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
Pre-Capture PCR Master Mix	Column 5 (A5-H5)	22 µL	33 µL	44 µL	55 µL	77 µL	143 µL

Table 161 Sample Buffer source plate for TS_D1000 protocol - used on page 74

Solution	Position on Source Plate	Volume of Sample Buffer 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
D1000 Sample Buffer	Column 1 (A1-H1)	11 µL	17 µL	23 µL	29 µL	41 µL	77 µL

Fast Hybridization

Table 162 Block master mix for fast hybridization - used on page 98

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	32 µL	53 µL	74 µL	96 µL	138 µL	276 µL
SureSelect XT HS2 Blocker Mix (blue cap)	5.0 µL	64 µL	106 µL	149 µL	191 µL	276 µL	553 µL
Total Volume	**7.5 µL**	**96 µL**	**159 µL**	**223 µL**	**287 µL**	**414 µL**	**829 µL**

Table 163 Probe master mix for Probes <3 Mb, 8 rows of wells, fast hybridization - used on page 98

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 µL	149 µL	208 µL	268 µL	402 µL	818 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	15 µL	19 µL	29 µL	58 µL
SureSelect Fast Hybridization Buffer (bottle)	6.0 µL	77 µL	128 µL	179 µL	230 µL	344 µL	701 µL
Probe (with design <3.0 Mb)	2.0 µL	26 µL	43 µL	60 µL	77 µL	115 µL	234 µL
Total Volume	**15.5 µL**	**198.4 µL**	**330.6 µL**	**462 µL**	**593 µL**	**890 µL**	**1811 µL**

Table 164 Probe master mix for Probes ≥3 Mb, 8 rows of wells, fast hybridization - used on page 99

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 µL	51 µL	85 µL	119 µL	153 µL	230 µL	468 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	15 µL	19 µL	29 µL	58 µL
SureSelect Fast Hybridization Buffer (bottle)	6 µL	77 µL	128 µL	179 µL	230 µL	344 µL	701 µL
Probe (with design ≥3 Mb)	5 µL	64 µL	106 µL	149 µL	191 µL	287 µL	584 µL
Total Volume	**15.5 µL**	**198.4 µL**	**329.6 µL**	**462 µL**	**593 µL**	**890 µL**	**1811 µL**

Table 165 Probe master mix for Probes <3 Mb, single row of wells, fast hybridization - used on page 99

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	11 µL	18 µL	25 µL	32 µL	49 µL	98 µL
RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.3 µL	3.5 µL	7 µL
SureSelect Fast Hybridization Buffer (bottle)	6 µL	9 µL	15 µL	21 µL	27 µL	42 µL	84 µL
Probe (with design <3.0 Mb)	2 µL	3 µL	5 µL	7 µL	9 µL	14 µL	28 µL
Total Volume	**15.5 µL**	**23.8 µL**	**39.3 µL**	**54.8 µL**	**70.3 µL**	**108.5 µL**	**217 µL**

Table 166 Probe master mix for Probes ≥3 Mb, single row of wells, fast hybridization - used on page 99

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 µL	6 µL	10 µL	14 µL	18 µL	28 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 µL
SureSelect Fast Hybridization Buffer (bottle)	6 µL	9 µL	15 µL	21 µL	27 µL	42 µL	84 µL
Probe (with design ≥3 Mb)	5 µL	8 µL	13 µL	18 µL	23 µL	35 µL	70 µL
Total Volume	**15.5 µL**	**23.8 µL**	**38.3 µL**	**54.8 µL**	**70.3 µL**	**108.5 µL**	**217 µL**

Table 167 Master mix source plate for fast hybridization protocol Hyb_XT_LI_ILM - used on page 100

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**
Block master mix	Column 1 (A1-H1)	11 µL	19 µL	27 µL	35 µL	51 µL	103 µL
Probe Hybridization master mix	Column 2 (A2-H2)	23 µL	39 µL	54 µL	70 µL	109 µL	217 µL

Overnight Hybridization

Table 168 Block master mix for overnight hybridization - used on page 121

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	32 µL	53 µL	74 µL	96 µL	138 µL	276 µL
SureSelect XT HS2 Blocker Mix (blue cap)	5 µL	64 µL	106 µL	149 µL	191 µL	276 µL	553 µL
Total Volume	**7.5 µL**	**96 µL**	**159 µL**	**223 µL**	**287 µL**	**414 µL**	**829 µL**

Table 169Overnight Hybridization Buffer - used on page 122

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Hyb 1 (bottle)	6.63 µL	90 µL	149 µL	209 µL	269 µL	403 µL	820 µL
SureSelect Hyb 2 (red cap)	0.27 µL	3.6 µL	6.1 µL	8.5 µL	10.9 µL	16.4 µL	33 µL
SureSelect Hyb 3 (yellow cap)	2.65 µL	36 µL	60 µL	83 µL	107 µL	161 µL	328 µL
SureSelect Hyb 4 (black cap)	3.45 µL	47 µL	78 µL	109 µL	140 µL	210 µL	427 µL
Total Volume	**13.0 µL**	**176.6 µL**	**293.1 µL**	**409.5 µL**	**526.9 µL**	**790.4 µL**	**1608 µL**

Table 170 Probe master mix for Probes <3 Mb, 8 rows of wells, overnight hybridization - used on page 122

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	57 µL	96 µL	134 µL	172 µL	258 µL	526 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	15 µL	19 µL	29 µL	58 µL
Overnight Hybridization Buffer (from **page 121**)	13 µL	166 µL	276 µL	387 µL	497 µL	746 µL	1519 µL
Probe (with design <3.0 Mb)	2 µL	26 µL	43 µL	60 µL	77 µL	115 µL	234 µL
Total Volume	**20 µL**	**255.4 µL**	**425.6 µL**	**596 µL**	**765 µL**	**1148 µL**	**2337 µL**

Table 171 Probe master mix for Probes ≥3 Mb, 8 rows of wells, overnight hybridization - used on page 123

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	19 µL	32 µL	45 µL	57 µL	86 µL	175 µL
RNase Block (purple cap)	0.5 µL	6.4 µL	10.6 µL	15 µL	19 µL	29 µL	58 µL
Overnight Hybridization Buffer (from **step 9**)	13 µL	166 µL	276 µL	387 µL	497 µL	746 µL	1519 µL
Probe (with design ≥3.0 Mb)	5 µL	64 µL	106 µL	149 µL	191 µL	287 µL	584 µL
Total Volume	**20 µL**	**255.4 µL**	**424.6 µL**	**496 µL**	**764 µL**	**1148 µL**	**2336 µL**

Table 172 Probe master mix for Probes <3 Mb, single row of wells, overnight hybridization - used on page 123

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	6.8 µL	11.3 µL	15.8 µL	20 µL	31 µL	63 µL
RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.3 µL	3.4 µL	7 µL
Overnight Hybridization Buffer (from **step 9**)	13 µL	20 µL	33 µL	46 µL	59 µL	90 µL	183 µL
Probe (with design <3 Mb)	2 µL	3 µL	5 µL	7 µL	9 µL	14 µL	28 µL
Total Volume	**20 µL**	**30.6 µL**	**50.6 µL**	**70.6 µL**	**90.3 µL**	**138.4 µL**	**281 µL**

Table 173 Probe master mix for Probes ≥3 Mb, single row of wells, overnight hybridization - used on page 123

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	2.3 µL	3.8 µL	5.3 µL	7 µL	10 µL	21 µL
RNase Block (purple cap)	0.5 µL	0.8 µL	1.3 µL	1.8 µL	2.3 µL	3.4 µL	7 µL
Overnight Hybridization Buffer (from **step 9**)	13 µL	20 µL	33 µL	46 µL	59 µL	90 µL	183 µL
Probe (with design ≥3 Mb)	5 µL	8 µL	13 µL	18 µL	23 µL	34 µL	70 µL
Total Volume	**20 µL**	**31.1 µL**	**51.1 µL**	**71.1 µL**	**91.3 µL**	**137.4 µL**	**281 µL**

Table 174 Master mix source plate for overnight hybridization protocol Hyb_XT_LI_ILM - used on page 124

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
Block master mix	Column 1 (A1-H1)	11 µL	19 µL	27 µL	35 µL	51 µL	103 µL
Probe Hybridization master mix	Column 2 (A2-H2)	30 µL	50 µL	70 µL	90 µL	138 µL	281 µL

Hybrid Capture and Washing

Table 175 Magnetic bead washing mixture - used on page 131

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 176 Resuspension of magnetic beads - used on page 131

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

Post-Capture PCR

Table 177 Post-Capture PCR master mix - used on page 137

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 µL	26 µL	34 µL	43 µL	57 µL	111 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	17 µL	26 µL	34 µL	43 µL	57 µL	111 µL
Total Volume	**12 µL**	**204 µL**	**307 µL**	**408 µL**	**511 µL**	**688 µL**	**1327 µL**

Table 178 Master mix source plate for post-capture PCR protocol Post-CapPCR_XT_LI_ILM - used on page 137

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
Post-Capture PCR Master Mix	Column 1 (A1-H1)	23 µL	36 µL	49 µL	62 µL	82 µL	163 µL

Table 179 Sample Buffer Source Plate for TS_HighSensitivity_D1000 protocol - used on page 145

Solution	Position on Source Plate	Volume of Sample Buffer 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
High Sensitivity D1000 Sample Buffer	Column 2 (A2-H2)	8 µL	11 µL	14 µL	17 µL	23 µL	44 µL

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 180 Genomic DNA Input Volumes

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

Table 181 XT HS2 Index Primer Pairs Volume on Primer Plate

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

Table 182 AMPure XP Bead Volumes for AMPure XP Protocols

Protocol or Runset	Volume of AMPure Beads per Well*
LibraryPrep_XT_HS2_ILM	80 µL
AMPureXP_XT_HS2_ILM (Pre-Cap PCR - SinglePlex)	50 µL
AMPureXP_XT_HS2_ILM (Pre-Cap PCR - MultiPlex)	50 µL
AMPureXP_XT_HS2_ILM (Concentration of Pool)	180 µL
AMPureXP_XT_HS2_ILM (Post-Capture PCR)	50 µL

* When preparing the plates of AMPure XP beads, fill the columns of the reservoir with enough of the bead suspension to cover the pyramid-shaped wells

Table 183 Water and Ethanol Volumes for AMPure XP Protocols

Reagent	Volume per Reservoir
70% ethanol in Agilent deep well reservoir	50 mL
Nuclease-free water in Agilent shallow well reservoir	30 mL

Troubleshooting Guide

If recovery of gDNA from samples is low

Using excess tissue for gDNA isolation can reduce yield. Use only the amount of each specific tissue type recommended by the gDNA isolation protocol.

Tissue sample lysis may not have been optimal during gDNA isolation. Monitor the extent of sample lysis during the Proteinase K digestion at 56°C by gently pipetting the digestion reaction every 20–30 minutes, visually inspecting the solution for the presence of tissue clumps. If clumps are still present after the 1-hour incubation at 56°C, add another 10 µL of Proteinase K and continue incubating at 56°C, with periodic mixing and visual inspections, for up to two additional hours. When the sample no longer contains clumps of tissue, move the sample to room temperature until lysis is complete for the remaining samples. Do not over-digest. Individual samples may be kept at room temperature for up to 2 hours before resuming the protocol. Do not exceed 3 hours incubation at 56°C for any sample.

If yield of pre-capture libraries is low

The library preparation protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance of the highly viscous buffer and enzyme solutions used in the protocol. Be sure to adhere to all instructions when setting up the reactions.

PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the pre-capture PCR cycle number by 1 to 2 cycles. If a high molecular weight peak (>500 bp) is observed in the electropherogram for a sample with low yield, the DNA may be overamplified. Repeat library preparation for the sample, decreasing the pre-capture PCR cycle number by 1 to 3 cycles.

DNA isolated from degraded samples, including FFPE tissue samples, may be over-fragmented or have modifications that adversely affect library preparation processes. Use the Agilent NGS FFPE QC Kit to determine the precise quantity of amplifiable DNA in the sample and allow direct normalization of input DNA amount.

Performance of the solid-phase reversible immobilization (SPRI) purification step may be poor. Verify the expiration date for the vial of AMPure XP beads used for purification. Adhere to all bead storage and handling conditions recommended by the manufacturer. Ensure that the beads are kept at room temperature for at least 30 minutes before use. Use freshly-prepared 70% ethanol for each SPRI procedure.

If solids observed in the End Repair-A Tailing Buffer

Vortex the solution at high speed until the solids are dissolved. The observation of solids when first thawed does not impact performance, but it is important to mix the buffer until all solutes are dissolved.

If pre-capture library fragment size is larger than expected in electropherograms

Shearing may not be optimal. For intact, high-quality DNA samples, ensure that mechanical shearing is completed using the two-round shearing protocol provided, including all spinning and vortexing steps.

When using the mechanical shearing method, any bubbles present on the microTUBE filament may disrupt complete shearing. Spin the microTUBE for 30 seconds before the first round of shearing to ensure that any bubbles are released.

If pre-capture library fragment size is different than expected in electropherograms

FFPE DNA pre-capture libraries may have a smaller fragment size distribution due to the presence of DNA fragments in the input DNA that are smaller than the target DNA shear size.

DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. When preparing the AMPure XP bead plate for pre-capture purification, mix the beads until the suspension appears homogeneous and consistent in color before adding the bead suspension to the shallow well reservoir. After preparation of the AMPure XP bead plate, seal the plate and store at 4°C until needed.

If low molecular weight adaptor-dimer peak is present in pre-capture library electropherograms

The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to the samples analyzed on **page 78**. 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 appropriate AMPureXP_XT_HS2_ILM (Pre-Cap 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 98**, and that solutions containing the Probe are not held at room temperature for extended periods.

If post-capture library fragment size is different than expected in electropherograms

DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. When preparing the AMPure XP bead plate for post-capture purification, mix the beads until the suspension appears homogeneous and consistent in color before adding the bead suspension to the shallow well reservoir. After preparation of the AMPure XP bead plate, seal the plate and store at 4°C until needed.

If low % on-target is observed in library sequencing results

Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a thermal cycler in close proximity to the NGS Workstation to retain the 65°C sample temperature during transfer step (**step 27** on **page 104** for fast hybridization; **step 28** on **page 127** for overnight hybridization).

If low uniformity of coverage with high AT-dropout is observed in library sequencing results

High AT-dropout may indicate that hybridization conditions are too stringent to obtain the desired level of coverage for AT-rich targets.

- For libraries target-enriched using the SureSelect XT HS Human All Exon V8-based probes or SureSelect XT HS Clinical Research Exome V4 probe using the fast hybridization program in **Table 66** on page 96 (including segment with one-hour incubation at 65°C), repeat target enrichment using the hybridization program in **Table 67** on page 96 (without the one-hour incubation at 65°C segment). For all other probes, repeat target enrichment at lower stringency using a modified thermal cycler program for hybridization, reducing the hybridization temperature in segments 4 and 5 from 65°C to 62.5°C or 60°C (see **Table 67** on page 96).
- For libraries target-enriched using the SureSelect XT HS Human All Exon V8-based probes or SureSelect XT HS Clinical Research Exome V4 probe using the overnight hybridization program in **Table 83** on page 120, reduce the temperature in Segment 2 through Segment 5 from 67.5°C to 66°C.

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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).

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