SureSelect XT HS2 RNA Kits

Automated using Agilent NGS Bravo Option A

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

Protocol

Version A0, February 2023

SureSelect platform manufactured with Agilent SurePrint technology.

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

Notices

Manual Part Number

G9993-90020

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Acknowledgment

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CAUTION

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In this Guide...

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

1 Before You Begin

This chapter contains information that you should read and understand before you start an experiment.

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

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

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.

4 Preparation of Input RNA and Conversion to cDNA

This chapter describes the steps to prepare input RNA samples, including RNA fragmentation when required, and the steps to convert the RNA fragments to strand-specific cDNA prior to sequencing library preparation and target enrichment.

5 Library Preparation

This chapter describes the steps for the automated preparation of cDNA NGS libraries for sequencing using the Illumina paired-read platform.

6 Hybridization

This chapter describes the steps to hybridize and capture the prepared DNA library using a SureSelect or ClearSeq Probe Capture Library.

7 Post-Capture Sample Processing for Multiplexed Sequencing

This chapter describes the steps for post-capture amplification and sequencing sample preparation guidelines.

8 Reference

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

Content

1 Before You Begin

Make sure you have the most current protocol. Go to **www.agilent.com** and search for G9993-90020.

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

NOTE

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

For automated sample processing procedures using Agilent NGS Workstation Option B, see publication G9993-90010.

For non-automated sample processing procedures, see publication G9989-90000 (for post-capture pooling workflow) or G9993-90000 (for pre-capture pooling workflow).

NOTE

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

Procedural Notes

- Certain protocol steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the Agilent NGS Bravo to allow rapid and efficient plate transfer.
- If your thermal cycler is compatible with the use of compression pads, add a compression pad whenever you load a plate that was sealed with the PlateLoc thermal microplate sealer. The pad improves contact between the plate and the heated lid of the thermal cycler.
- 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

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

Materials Required

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

- See **Table 1** and **Table 2** for the reagents and equipment required for all workflow types.
- See **Table 3** for the SureSelect XT HS2 RNA Reagent Kits. The table includes kits that are suitable for workflows with pre-capture pooling of cDNA libraries and kits suitable for workflows with post-capture pooling of cDNA libraries.
- See **Table 4** for compatible probes. The table distinguishes between probes suitable for workflows with pre-capture pooling of cDNA libraries and probes suitable for workflows with post-capture pooling of cDNA libraries.
- See **Table 5** for nucleic acid analysis platform options.

Table 1 Required Reagents--All 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
QPCR Human Reference Total RNA –for use as control input RNA (optional)	Agilent p/n 750500
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

* Separate purchase **not** required when using the SureSelect XT HS2 RNA Reagent Kits that include SureSelect RNA AMPure® XP Beads and SureSelect Streptavidin Beads (Agilent p/n G9992A, G9992B, G9992C, or G9992D). See **Table 3**.

Table 2 Required Equipment--All Workflow Types

Description	Vendor and Part Number
Agilent NGS Bravo Option A Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5573AA (VWorks software version 13.1.0.1366)
Agilent PlateLoc Thermal Microplate Sealer	Agilent p/n G5585HA or G5585BA
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Thermal cycler and accessories (including compression pads, if compatible)	Various suppliers *Important*: Not all PCR plate types are supported for use in the VWorks automation protocols for the Agilent NGS Bravo. Select a thermal cycler that is compatible with one of the supported PCR plate types. See supported plate types in the listing below.
PCR plates compatible with the Agilent NGS Bravo and associated VWorks automation protocols	Only the following PCR plates are supported: • 96 ABI PCR half-skirted plates (MicroAmp Optical plates), Thermo Fisher Scientific p/n N8010560 • 96 Agilent semi-skirted PCR plate, Agilent p/n 401334 • 96 Eppendorf twin.tec half-skirted PCR plates, Eppendorf p/n 951020303 • 96 Eppendorf twin.tec PCR plates (full-skirted), Eppendorf p/n 951020401 or 951020619 • 96 Armadillo PCR plates (full-skirted), Thermo Fisher Scientific p/n AB2396
Eppendorf twin.tec full-skirted 96-well PCR plates, or Armadillo PCR plates, 96-wells (full-skirted)	Eppendorf p/n 951020401 or 951020619 Thermo Fisher Scientific p/n AB2396
Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 19 mm height – used when Bravo setup calls for **Agilent Shallow Well Reservoir**	Agilent p/n 201254-100
Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 44 mm height – used when Bravo setup calls for **Agilent Deep Well Reservoir**	Agilent p/n 201244-100
Agilent Storage/Reaction Microplates, 96 wells, 1 mL/round well – used when Bravo setup calls for **Agilent DeepWell Plate** or **Agilent DW Plate**	Agilent p/n 203426-100
Agilent Storage/Reaction Microplates, 96 wells, 2 mL/square well – used when Bravo setup calls for **Waste Plate (Agilent 2 mL Square Well)**	Agilent p/n 201240-100
Nucleic acid surface decontamination wipes	DNA Away Surface Decontaminant Wipes, Thermo Scientific p/n 7008, or equivalent
Low-adhesion 1.5-mL tubes (RNase, DNase, and DNA-free)	USA Scientific p/n 1415-2600
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

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

Description	Vendor and Part Number
Single channel pipettes (10-, 20-, 200-, and 1000-µL capacity)	Rainin Pipet-Lite Pipettes or equivalent
Sterile, nuclease-free aerosol barrier pipette tips	general laboratory supplier
Vortex mixer	general laboratory supplier
Ice bucket	general laboratory supplier
Powder-free gloves	general laboratory supplier

Table 3 Agilent SureSelect XT HS2 DNA Reagent Kits

Description	Agilent Part Number
For *Pre-Capture* Pooling	
SureSelect XT HS2 RNA Library Preparation Kit for ILM, 96 Reactions	G9993A (with Index Pairs 1–96) G9993B (with Index Pairs 97–192) G9993C (with Index Pairs 193–288) G9993D (with Index Pairs 289–384)
AND	
SureSelect XT HS2 RNA Target Enrichment Kit, 12 Hybs*	G9994A
For *Post-Capture* Pooling	
SureSelect XT HS2 RNA Reagent Kit, 96 Reactions *-- contains reagents for library preparation and target enrichment*	G9991A (with Index Pairs 1–96) G9991B (with Index Pairs 97–192) G9991C (with Index Pairs 193–288) G9991D (with Index Pairs 289–384)
OR	
SureSelect XT HS2 RNA Reagent Kit with AMPure® XP/Streptavidin Beads, 96 Reactions† *-- contains reagents for library preparation and target enrichment, and includes the necessary AMPure XP and Streptavidin beads*	G9992A (with Index Pairs 1–96) G9992B (with Index Pairs 97–192) G9992C (with Index Pairs 193–288) G9992D (with Index Pairs 289–384)

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

† AMPure, Beckman, and Beckman Coulter are trademarks or registered trademarks of Beckman Coulter, Inc.

Table 4 Compatible Probes based on Pooling Method

Probe Capture Library		Design Target	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 RNA 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			
	SureSelect Custom Tier2 0.5–2.9 Mb			
	SureSelect Custom Tier3 3–5.9 Mb			
	SureSelect Custom Tier4 6–11.9 Mb			
	SureSelect Custom Tier5 12–24 Mb			
Pre-designed Probes				
***Pre-Capture* Pooling**	SureSelect XT HS PreCap Human All Exon V8 (12 Hybs)**	Genome	S33266340	Agilent p/n 5191-6878
	SureSelect XT HS Pre-Cap Human All Exon V8 +UTR (12 Hybs)**	Genome	S33613271	Agilent p/n 5191-7406
	SureSelect Pre-Capture Pooling Human All Exon V7 (12 Hybs)**	Genome	S31285117	Agilent p/n 5191-5735
	SureSelect XT2 Clinical Research Exome V2 (12 Hybs)**	Genome	S30409818	Agilent p/n 5190-9501
	SureSelect XT2 Mouse All Exon (12 Hybs)**	Genome	S0276129	Agilent p/n 5190-4682
	ClearSeq Inherited Disease XT2 (12 Hybs)**	Genome	S0684402	Agilent p/n 5190-7525
	ClearSeq Comprehensive Cancer XT2 (6 Hybs)†	Genome	0425761	Agilent p/n 5190-8018
***Post-Capture* Pooling**	SureSelect XT HS Human All Exon V8, 96 Reactions	Genome	S33266340	Agilent p/n 5191-6875
	SureSelect XT HS Human All Exon V8+UTR, 96 Reactions	Genome	S33613271	Agilent p/n 5191-7403
	SSel XT HS and XT Low Input Human All Exon V7, 96 Reactions	Genome	S31285117	Agilent p/n 5191-4029
	SureSelect XT Clinical Research Exome V2, 96 Reactions	Genome	S30409818	Agilent p/n 5190-9492
	SureSelect XT Mouse All Exon, 96 Reactions	Genome	S0276129	Agilent p/n 5190-4642
	ClearSeq Comprehensive Cancer XT, 96 Reactions	Genome	0425761	Agilent p/n 5190-8012
	ClearSeq Inherited Disease XT, 96 Reactions	Genome	S0684402	Agilent p/n 5190-7519
	ClearSeq RNA Kinome, 96 Reactions	Transcriptome	0320691	Agilent p/n 5190-4802

Table 4 Compatible Probes based on Pooling Method (continued)

Probe Capture Library		Design Target	Design ID	Ordering Information
Pre-designed Probes customized with additional *Plus* custom content				
***Pre-Capture* Pooling**	SureSelect XT2 Clinical Research Exome V2 Plus 1 (12 Hybs)**	Genome		
	SureSelect XT2 Clinical Research Exome V2 Plus 2 (12 Hybs)**	Genome		
	ClearSeq Comprehensive Cancer Plus XT2 (6 Hybs)†	Genome		
	ClearSeq Inherited Disease Plus XT2 (12 Hybs)**	Genome		
***Post-Capture* Pooling**	SSel XT HS and XT Low Input Human All Exon V7 Plus 1	Genome		
	SSel XT HS and XT Low Input Human All Exon V7 Plus 2	Genome		
	SureSelect XT Clinical Research Exome V2 Plus 1	Genome		
	SureSelect XT Clinical Research Exome V2 Plus 2	Genome		
	ClearSeq Comprehensive Cancer Plus XT	Genome		
	ClearSeq Inherited Disease Plus XT	Genome		

Please visit the SureDesign website to design the customized *Plus* content and obtain ordering information. Contact the SureSelect support team (see **page 2**) or your local representative if you need assistance.

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

† The 6-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 16 samples/pool (for 6 hybs × 16 samples/hyb). Contains enough reagent for 6 hybridization reactions using the run setup on **page 106**. The hybridization protocol can only process columns in which all 8 wells contain a hybridization reaction. In order to have enough reagent to run 3 full columns (i.e., 24 wells), you must purchase 4 units of the 6-Hyb Probe.

‡ The 30-Hyb Probes support target enrichment for 480 samples pooled using the configuration recommended for the included designs of 16 samples/pool (for 30 hybs × 16 samples/hyb). Contains enough reagent for 30 hybridization reactions using the run setup on **page 106**. The hybridization protocol can only process columns in which all 8 wells contain a hybridization reaction.

** The 12-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 8 samples/pool (for 12 hybs × 8 samples/hyb). Contains enough reagent for 6 hybridization reactions using the run setup on **page 106**. The hybridization protocol can only process columns in which all 8 wells contain a hybridization reaction. In order to have enough reagent to run 3 full columns (i.e., 24 wells), you must purchase 2 units of the 12-Hyb Probe.

Table 5 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

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

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

About the NGS Bravo Option A

CAUTION Before you begin, make sure that you have read and understand operating, maintenance and safety instructions for using the Bravo platform and additional devices and software. Refer to the user guides listed in **Table 6**.

Review the user guides listed in **Table 6** (available at Agilent.com) to become familiar with the general features and operation of the automation components. Instructions for using the Bravo platform and other automation components in the SureSelect XT HS2 Target Enrichment workflow are detailed in this user guide.

Table 6 Agilent NGS Bravo User Guide reference information

Device	User Guide part number
Bravo Platform	SD-V1000376 (previously G5562-90000)
VWorks Software (version 13.1.0.1366)	G5415-90068
PlateLoc Thermal Microplate Sealer	G5585-90010

About the Bravo Platform

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

Bravo Platform Deck

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

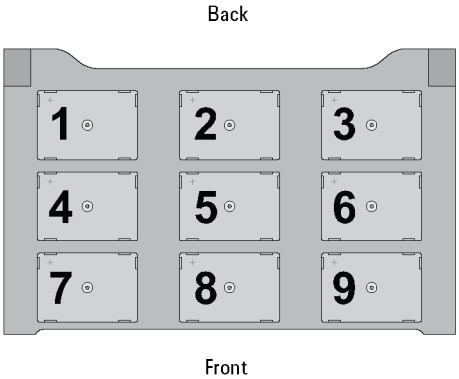


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

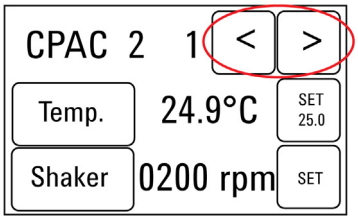
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

Bravo deck heat block temperatures may be changed using the Inheco Multi TEC Control device touchscreen as described in the steps below. See **Table 7** for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

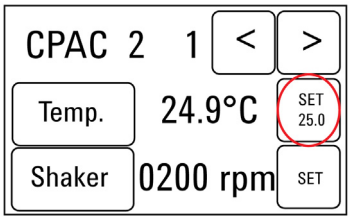
Table 7 Inheco Multi TEC Control touchscreen designations

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

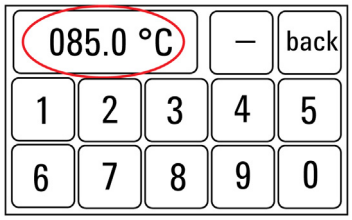
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



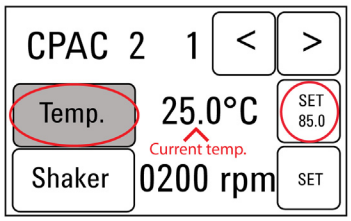
2 To set the temperature of the selected block, press the SET button.



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



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



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

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

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

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

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

3 Press the **START** button.

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

VWorks Automation Control Software

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

NOTE

The instructions in this manual are compatible with VWorks software version 13.1.0.1366.

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

Logging in to the VWorks software

1 Double-click the VWorks icon or the RNA_XT_HS2_ILM_v.Ax.x.x.VWForm shortcut (where x.x.x is the version number) on the Windows desktop to start the VWorks software.

2 If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.

3 In the User Authentication dialog, type your VWorks user name and password, and click **OK**. (If no user account is set up, contact the administrator.)

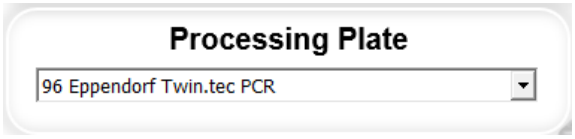
Using the RNA_XT_HS2_ILM_v.Ax.x.x.VWForm to setup and start a run

Use the VWorks form RNA_XT_HS2_ILM_v.Ax.x.x.VWForm (where x.x.x is the version number), shown below, to set up and start each SureSelect automation protocol.

1 Open the form using the RNA_XT_HS2_ILM_v.Ax.x.x.VWForm shortcut on your desktop.

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

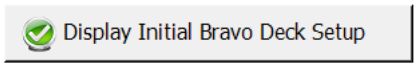
The processing plate is either a 96-well Eppendorf twin.tec plate (Eppendorf p/n 951020401 or 951020619) or a 96-well Armadillo plate (Thermo Fisher Scientific p/n AB2396).



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

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

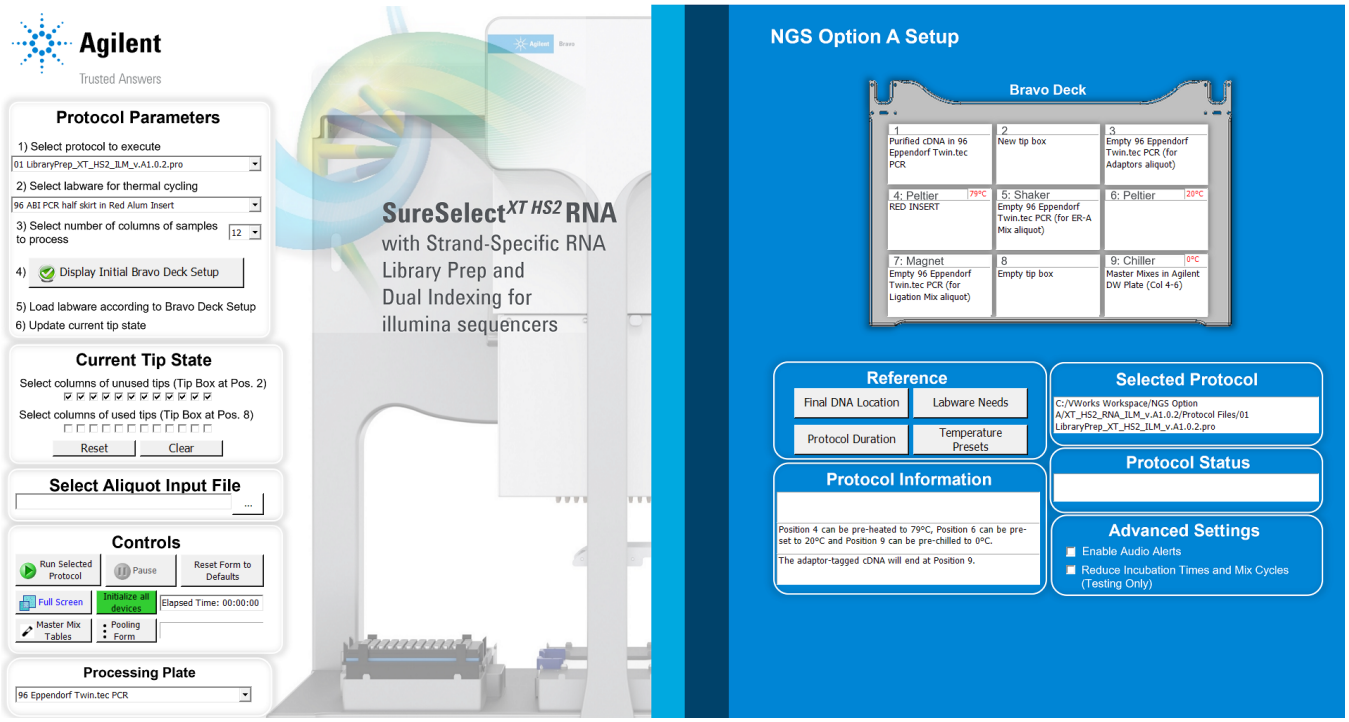
4 Once all run parameters have been specified on the form, click **Display Initial Bravo Deck Setup**.



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

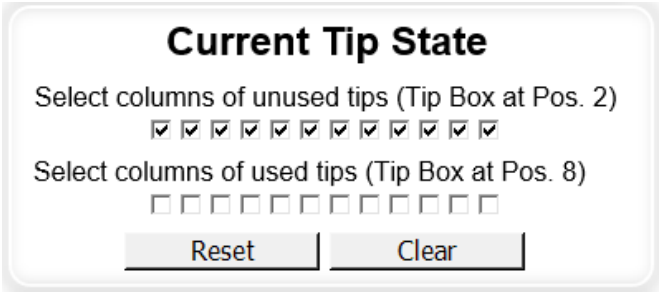
Load the Bravo Deck with labware and reagents as specified in the **Bravo Deck** graphic in the top right quadrant of the form.

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



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

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



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

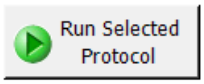
For an empty tip box, all positions of the **Current Tip State** used tip indicator (bottom portion, Box 8) should be cleared, as shown above. Clicking **Reset** clears all columns for position 8.

NOTE

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

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

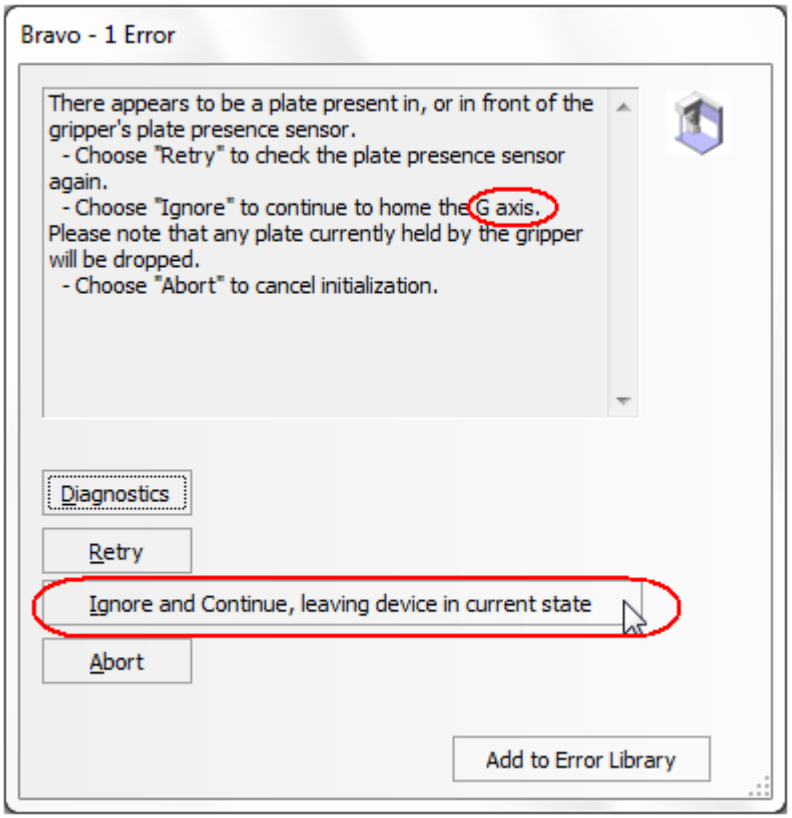
7 After verifying that the NGS Bravo has been set up correctly, click **Run Selected Protocol**.



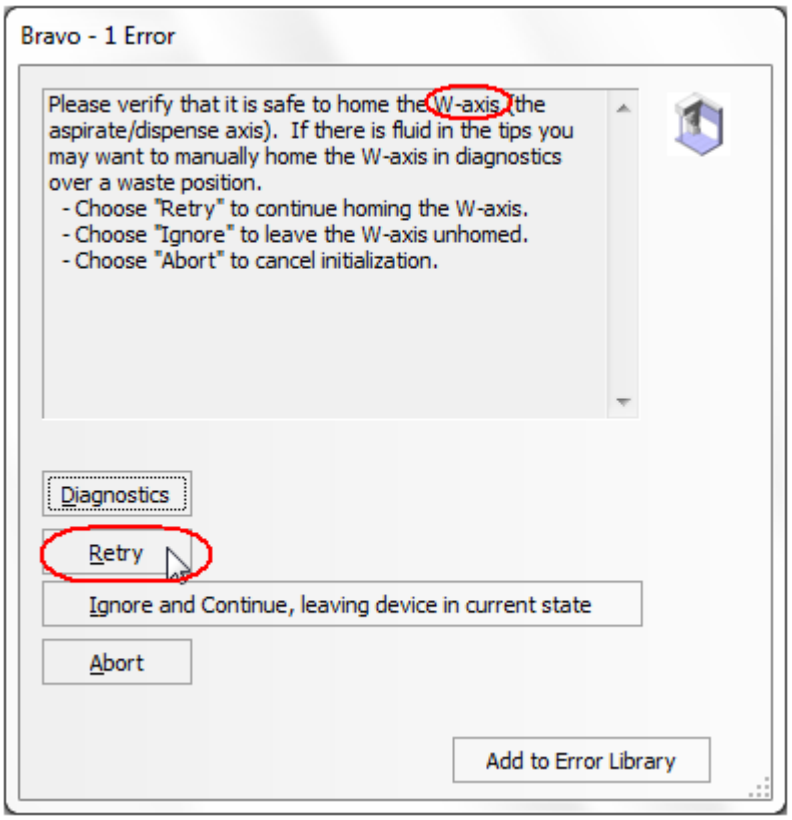
Error messages encountered at start of run

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

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



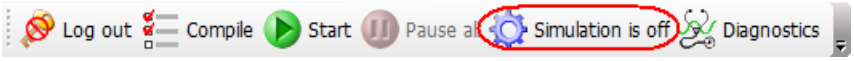
2 If you encounter the W-axis error message shown below, select **Retry**.



Verifying the Simulation setting

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

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



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

NOTE

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

Overview of the SureSelect Target Enrichment Procedure

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

Agilent offers four different plates of index pairs for use with the SureSelect XT HS2 RNA library preparation reagents to allow for multiplexed sequencing (refer to **"Index Primer Pair Plate Maps"** on page 161). Depending on the SureSelect XT HS2 RNA 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 9** for a summary of the VWorks protocols used during the workflow. Then, see **Preparation of AMPure XP Bead Plates**, **Library Preparation**, **Hybridization**, and **Post-Capture Sample Processing for Multiplexed Sequencing** chapters for complete instructions for use of the VWorks protocols for sample processing.

The SureSelect XT HS2 RNA library preparation protocol is compatible with both high-quality total RNA prepared from fresh or fresh frozen samples and lower-quality RNA prepared from FFPE samples. The RNA input quantity ranges from 10 to 200 ng RNA, depending on RNA quality.

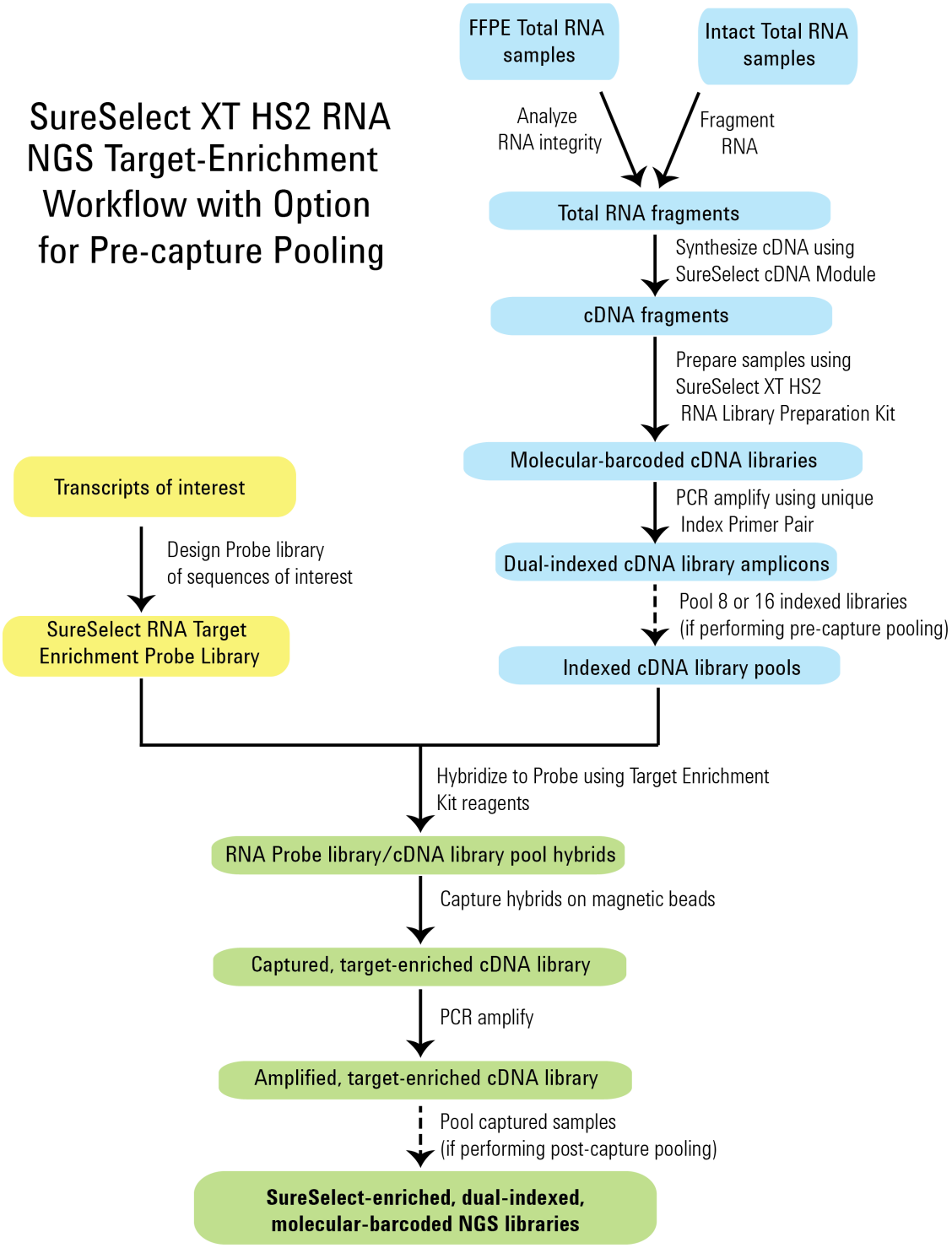


Figure 2 Overall sequencing sample preparation workflow.

Workflow Modulations

The SureSelect XT HS2 RNA target enrichment workflow can be modulated for different applications as described below and summarized in **Table 8** on page 29.

RNA Sample Integrity Protocols are compatible with both high-quality, intact total RNA prepared from fresh or fresh frozen samples and lower-quality total RNA prepared from FFPE samples with minor protocol modifications. In particular, intact RNA requires heat fragmentation prior to cDNA synthesis while FFPE RNA is already sufficiently fragmented.

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

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

Table 8 Summary of workflow modulations supported by the automation protocols

Property	Options	Usage Notes
RNA Sample Integrity	Intact RNA	After the automated fragmentation protocol, transfer the plate of RNA samples to a thermal cycler for heat fragmentation at 94°C as described in **Table 20** on page 50.
	FFPE RNA	Qualify RNA before use in assay; see **"Assess initial quality of RNA samples (FFPE RNA only)"** on page 48. After the automated fragmentation protocol, do not transfer the plate to a thermal cycler for heat fragmentation.
Pooling Strategy	Pre-Capture Pooling	For library preparation, use a SureSelect XT HS2 RNA Library Preparation Kit that is compatible with pre-capture pooling (e.g., Agilent part numbers G9993A through G9993D). For target enrichment, use the SureSelect XT HS2 RNA Target Enrichment Kit. Refer to **Table 3** on page 15.
	Post-Capture Pooling	Use one of the SureSelect XT HS2 RNA Reagent Kits compatible with post-capture pooling (Agilent part numbers G9991A through G9991D, or G9992A through G9992D). Kits include reagents for both library preparation and target enrichment. Refer to **Table 3** on page 15.

Automation Protocols used in the Workflow

Table 9 Overview of VWorks protocols

Workflow Step	Substep	VWorks Protocols Used for NGS Bravo automation
AMPure XP Bead Aliquoting	Aliquot AMPure XP beads for use in the Second-Strand cDNA cleanup protocol	AMPureXP_Aliquot_cDNA
	Aliquot AMPure XP beads for use in the Library Prep cleanup protocol	AMPureXP_Aliquot_LibPrep
	Aliquot AMPure XP beads for use in the Pre-Capture PCR cleanup protocol	AMPureXP_Aliquot_PreCap
	Aliquot AMPure XP Beads for use in the Pre-Capture Pooling protocol for concentrating the DNA	AMPureXP_Aliquot_Pooling
	Aliquot AMPure XP Beads for use in the Post-Capture PCR cleanup protocol	AMPureXP_Aliquot_PostCap
RNA Preparation and cDNA Conversion	Mix RNA samples with the 2X Priming Buffer	00a RNA_Frag_XT_HS2_ILM
	Synthesize first-strand cDNA	00b RNA_FirstStrand_XT_HS2_ILM
	Synthesize second-strand cDNA	00c RNA_SecondStrand_XT_HS2_ILM
	Purify cDNA using AMPure XP beads	00d Cleanup_cDNA_XT_HS2_ILM
Library Preparation	Prepare duplex, molecular-barcoded DNA libraries	01 LibraryPrep_XT_HS2_ILM
	Purify DNA libraries using AMPure XP beads	02 Cleanup_LibPrep_XT_HS2_ILM
	Amplify indexed DNA libraries with unique dual indexing primer pair	03 Pre-CapPCR_XT_HS2_ILM
	Purify indexed DNA libraries using AMPure XP beads using an elution volume suitable for **single-plexed** hybridization (i.e., the post-capture pooling workflow)	04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM
	Purify indexed DNA libraries using AMPure XP beads using an elution volume suitable for **multi-plexed** hybridization (i.e., the pre-capture pooling workflow)	04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM
	Analyze indexed DNA libraries using Agilent TapeStation platform	05 TS_D1000
Library Pooling (for **pre-capture pooling** workflow)	Pool indexed DNA libraries in pools of 8 or 16	PreCapture_Pooling *This protocol is set up and executed from the XT_HS2_Pooling VWorks Form*
Single-Plex Pre-Hybridization (for **post-capture pooling** workflow)	Aliquot 500-1000 ng of prepped libraries	06a Aliquot_Libraries
Multi-Plex Pre-Hybridization (for **pre-capture pooling** workflow)	Dilute pooled samples of indexed cDNA libraries to normalize volumes to 100 µL	06b Aliquot_Water
	Concentrate pooled samples to 24 µL for hybridization	06c PoolingConcentration_XT_HS2_ILM

Table 9 Overview of VWorks protocols

Workflow Step	Substep	VWorks Protocols Used for NGS Bravo automation
Hybridization and Capture	Hybridize prepped libraries or library pools to Probe (target enrichment)	07 Hyb_XT_HS2_ILM
	Capture cDNA hybrids	08 SSELCapture_XT_HS2_ILM
	Wash cDNA hybrids	09 SSELWash_XT_HS2_ILM
Post-Capture Sample Processing	Amplify target-enriched libraries or library pools	10 Post-CapPCR_XT_HS2_ILM
	Purify enriched, amplified libraries or library pools using AMPure XP beads	11 Cleanup_Post-CapPCR_XT_HS2_ILM
	Analyze final libraries or library pools using Agilent TapeStation platform	12 TS_HighSensitivity_D1000
	For post-capture pooling workflow, pool indexed cDNA libraries	13 Aliquot_Captures

Experimental Setup Considerations for Automated Runs

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

Table 10 Columns to Samples Equivalency

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

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

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

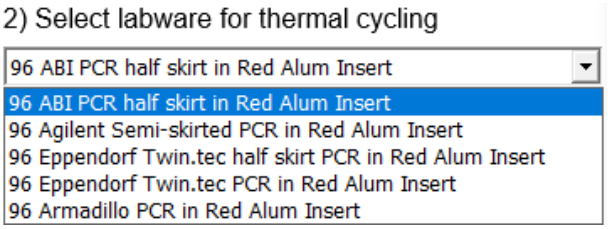
- The NGS Bravo processes samples column-wise beginning at column 1. Samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- Samples are indexed during pre-capture amplification (see **Figure 2**). Assign each sample to the appropriate indexing primer during experimental design, and place the sample in the well corresponding to its assigned primer. See **Table 99** on page 161 through **Table 102** on page 162 for indexing primer plate maps.
- 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 plate position 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 sizes of the captured targets. It is most efficient to process similar-sized Probes on the same plate. See **Table 70** on page 123 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 Bravo to allow rapid and efficient plate transfer.
- Several workflow steps require that the sample plate be sealed using the PlateLoc thermal microplate sealer and then centrifuged to collect any dispersed liquid. To maximize efficiency, locate the centrifuge in close proximity to the NGS Bravo.

PCR Plate Type Considerations

Automation protocols include several liquid-handling steps in which reagents are dispensed to PCR plates in preparation for transfer to a thermal cycler. For these steps, you must specify on the VWorks Form which PCR plate type will be used to allow correct configuration of the liquid handling components. Before you begin the automation protocol, make sure that you are using a supported PCR plate type. The PCR plate type to be used in the protocol is specified using the menu below. Vendor and part number information is provided for the supported plate types in **Table 11**.



CAUTION

The plates listed in **Table 11** are compatible with the Agilent NGS Bravo and associated VWorks automation protocols, designed to support use of various thermal cyclers.

Do not use PCR plates that are not listed in **Table 11**, even if they are compatible with your chosen thermal cycler.

Table 11 Ordering information for supported PCR plates

Description in VWorks menu	Vendor and part number
96 ABI PCR half-skirted plates (MicroAmp Optical plates)	Thermo Fisher Scientific p/n N8010560
96 Agilent semi-skirted PCR plate	Agilent p/n 401334
96 Eppendorf Twin.tec half-skirted PCR plates	Eppendorf p/n 951020303
96 Eppendorf Twin.tec PCR plates (full-skirted)	Eppendorf p/n 951020401
96 Armadillo PCR plates (full-skirted),	Thermo Fisher Scientific p/n AB2396

3 Preparation of AMPure XP Bead Plates

This chapter provides instructions on preparing all of the plates of AMPure XP beads that are needed throughout the entire workflow. Each plate of AMPure XP beads is prepared using a separate automation protocol available in the RNA_XT_HS2_ILM 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 cDNA purification

The **00d Cleanup_cDNA__XT_HS2_ILM** protocol requires a bead plate containing 105 µL of beads in each well. Use the **AMPureXP_Aliquot_cDNA** protocol to prepare the bead plate.

Prepare the NGS Bravo and reagents for protocol AMPureXP_Aliquot_cDNA

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.
   a. Verify that the AMPure XP bead suspension is at room temperature.
   b. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
   c. Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the **00d Cleanup_cDNA_XT_HS2_ILM** protocol (each column accommodates 8 samples).

Load the NGS Bravo

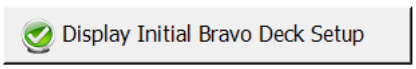
1. Load the Bravo deck according to **Table 12**.

Table 12 Initial Bravo deck configuration for protocol AMPureXP_Aliquot_cDNA

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

Run VWorks protocol AMPureXP_Aliquot_cDNA

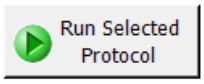
1. On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot_cDNA** protocol.
2. Select the number of columns of samples to be processed.
3. Click **Display Initial Bravo Deck Setup**.



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

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

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



Running the AMPureXP_Aliquot_cDNA protocol takes approximately 5 minutes. The protocol directs the NGS Bravo to aliquot 105 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent Deep Well plate.

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

8 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the **00d Cleanup_cDNA_XT_HS2_ILM** protocol (refer to **Table 32** on page 61). Use the plate within the next 24 hours.

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

Step 2. Prepare the bead plate to be used for library preparation

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

Prepare the NGS Bravo and reagents for protocol AMPureXP_Aliquot_LibPrep

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.
   a. Verify that the AMPure XP bead suspension is at room temperature.
   b. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
   c. Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the **02 Cleanup_LibPrep_XT_HS2_ILM** protocol (each column accommodates 8 samples).

Load the NGS Bravo

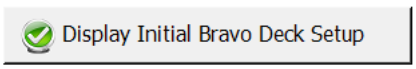
1. Load the Bravo deck according to **Table 13**.

Table 13 Initial Bravo deck configuration for protocol AMPureXP_Aliquot_LibPrep

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

Run VWorks protocol AMPureXP_Aliquot_LibPrep

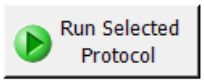
1. On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot_LibPrep** protocol.
2. Select the number of columns of samples to be processed.
3. Click **Display Initial Bravo Deck Setup**.



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

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

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



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

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

8 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the **02 Cleanup_LibPrep_XT_HS2_ILM** protocol (refer to **Table 36** 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 pre-capture purification

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

Prepare the NGS Bravo and reagents for protocol AMPureXP_Aliquot_PreCap

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.
   a. Verify that the AMPure XP bead suspension is at room temperature.
   b. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
   c. Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the pre-capture PCR cleanup protocol (each column accommodates 8 amplified cDNA samples).

Load the NGS Bravo

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

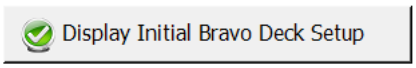
Table 14 Initial Bravo deck configuration for protocol AMPureXP_Aliquot_PreCap

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

Run VWorks protocol AMPureXP_Aliquot_PreCap

1. On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot_PreCap** protocol.
2. Select the number of columns of samples to be processed.

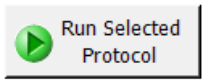
3 Click **Display Initial Bravo Deck Setup**.



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

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

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



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

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

8 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the **04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM** or **04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM** protocol (refer to **Table 43** on page 81). 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 concentration of pooled DNA (pre-capture pooling workflow only)

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

The bead plate for the **06c PoolingConcentration_XT_HS2_ILM** protocol is only needed if you are running the pre-capture pooling workflow option. If you are using the post-capture pooling workflow, proceed to **"Step 5. Prepare the bead plate to be used for post-capture purification"** on page 44.

Prepare the NGS Bravo and reagents for protocol AMPureXP_Aliquot_Pooling

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.
   a. Verify that the AMPure XP bead suspension is at room temperature.
   b. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
   c. Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the **06c PoolingConcentration_XT_HS2_ILM** protocol (each column accommodates 8 cDNA library pools).

Load the NGS Bravo

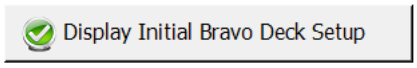
1. Load the Bravo deck according to **Table 15**.

Table 15 Initial Bravo deck configuration for protocol AMPureXP_Aliquot_Pooling

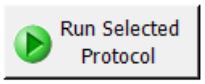
Location	Content
2	New tip box
5	Empty Agilent Deep Well Plate
6	Reservoir of AMPure XP bead suspension prepared in **step 2**, above
8	Empty tip box

Run VWorks protocol AMPureXP_Aliquot_Pooling

1. On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot_Pooling** protocol.
2. Select the number of columns of samples to be processed.
3. Click **Display Initial Bravo Deck Setup**.



4. Verify that the Bravo deck has been set up as displayed on the right side of the form.
5. Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.
6. When verification is complete, click **Run Selected Protocol**.



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

7. When the protocol is complete, remove the Agilent Deep Well plate containing the AMPure XP bead suspension from position 5 of the Bravo deck.
8. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the **06c PoolingConcentration_XT_HS2_ILM** protocol (refer to **Table 51** on page 101). 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 5. Prepare the bead plate to be used for post-capture purification

The **11 Cleanup_Post-CapPCR__XT_HS2_ILM** protocol requires a bead plate containing 50 µL of beads in each well. Use the **AMPureXP_Aliquot_PostCap** protocol to prepare the bead plate.

Prepare the NGS Bravo and reagents for protocol AMPureXP_Aliquot_PostCap

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.
   a. Verify that the AMPure XP bead suspension is at room temperature.
   b. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
   c. Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the **11 Cleanup_Post-CapPCR__XT_HS2_ILM** protocol (each column accommodates 8 indexed libraries).

Load the NGS Bravo

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

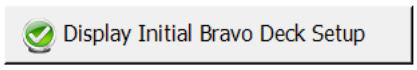
Table 16 Initial Bravo deck configuration for protocol AMPureXP_Aliquot_PostCap

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

Run VWorks protocol AMPureXP_Aliquot_PostCap

1. On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot_PostCap** protocol.
2. Select the number of columns of samples to be processed.

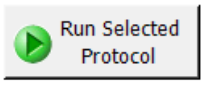
3 Click **Display Initial Bravo Deck Setup**.



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

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

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



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

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

8 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the **11 Cleanup_Post-CapPCR_XT_HS2_ILM** protocol (refer to **Table 75** on page 129). 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 Preparation of Input RNA and Conversion to cDNA

This chapter describes the steps to prepare input RNA samples, including RNA fragmentation when required, and the steps to convert the RNA fragments to strand-specific cDNA prior to sequencing library preparation and target enrichment.

The protocol is compatible with both intact RNA prepared from fresh or fresh frozen samples and lower-quality RNA prepared from FFPE samples. Make sure to read the instructions carefully as some steps differ depending on the RNA sample type.

RNA sequencing library preparation requires RNA fragments sized appropriately for the NGS workflow. In this section of the protocol, intact total RNA samples are chemically-fragmented by treatment with metal ions present in the 2X Priming Buffer at elevated temperature. FFPE-derived RNA samples are already sufficiently fragmented. The FFPE samples must be combined with the same 2X Priming Buffer, but the mixtures are held on ice, preventing further fragmentation of the FFPE-derived RNA.

Protocols in this section for both intact RNA and FFPE sample types are applicable to either 2 x 100 bp or 2 x 150 bp read-length sequencing.

The protocol steps in this section use the components listed in **Table 17**. Thaw and mix each component as directed in **Table 17** before use (refer to the *Where Used* column).

Table 17 Reagents thawed before use in protocol

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
2X Priming Buffer (tube with purple cap)	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	**page 50**
First Strand Master Mix (amber tube with amber cap)*	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice for 30 minutes then keep on ice	Vortexing	**page 55**
Second Strand Enzyme Mix (bottle)	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	**page 58**
Second Strand Oligo Mix (tube with yellow cap)	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	**page 58**

* The First Strand Master Mix contains actinomycin-D and is provided ready-to-use. Keep the reagent in the supplied amber vial to protect the contents from exposure to light.

Step 1. Prepare RNA samples

This step uses automation protocol **00a RNA_Frag_XT_HS2_ILM**.

Prepare total RNA from each sample in the run. The library preparation protocol requires 10 to 200 ng of total RNA in a 10 µL volume of nuclease-free water.

Consider preparing a run using a high-quality control RNA sample, such as Agilent's QPCR Human Reference Total RNA (p/n 750500). This control is especially recommended as the first run of the protocol to verify that all protocol steps are being successfully performed. Use of this control is also helpful for any required troubleshooting.

NOTE

Due to differences in the processing steps required for each sample type, Agilent recommends processing FFPE RNA samples and intact RNA samples in separate runs.

If you must combine sample types in the same run — for example, when including a control sample of Agilent QPCR Human Reference Total RNA with a set of FFPE RNA samples — then the sample types need to be separated into separate plasticware at the end of the **00a RNA_Frag_XT_HS2_ILM** protocol in order to accommodate the different processing steps (see **step 21** on **page 53**). Intact RNA samples should be transferred to a fresh PCR plate or tube before they are put in the thermal cycler to run the fragmentation program while the plate with the FFPE RNA samples is held on ice. At the end of the thermal cycling program, you can return the samples back to their original wells in the original PCR plate.

Assess initial quality of RNA samples (FFPE RNA only)

The quality assessment is only needed for FFPE-derived total RNA samples. If you are using intact (non-FFPE) RNA samples, proceed directly to **"Run the automated fragmentation protocol (both FFPE and intact RNA)"** on page 49.

For FFPE RNA, assessing the initial quality of each sample is necessary to determine the appropriate reaction conditions at several steps in the workflow. Use the steps below to qualify each FFPE RNA sample.

1 Use a small-volume spectrophotometer to determine sample absorbance at 260 nm, 280 nm, and 230 nm. Determine the RNA concentration and the 260/280 and 260/230 absorbance ratio values for the sample.

   High-quality RNA samples are indicated by values of approximately 1.8 to 2.0 for both ratios. Ratios with significant deviation from 2.0 indicate the presence of organic or inorganic contaminants, which may require further purification or may indicate that the sample is not suitable for use in RNA target enrichment applications.

2 Examine the starting size distribution of RNA in the sample using one of the RNA qualification systems described in **Table 18**. Select the specific assay appropriate for your sample based on the RNA concentration determined above in **step 1**.

Determine the DV200 (percentage of RNA in the sample that is >200 nt) using the analysis mode described in **Table 18**. RNA molecules must be >200 nucleotides for efficient conversion to cDNA library.

Table 18 RNA qualification platforms

Analysis Instrument	RNA Qualification Assay	Analysis to Perform
4200/4150 TapeStation	RNA ScreenTape or High Sensitivity RNA ScreenTape	Region analysis using TapeStation Analysis Software
2100 Bioanalyzer	RNA 6000 Pico Chip or NanoChip	Smear/Region analysis using 2100 Expert Software
5200 Fragment Analyzer	RNA Kit (15NT) or HS RNA Kit (15NT)	Analysis using ProSize Data Analysis Software

NOTE Grading of FFPE RNA quality by RNA Integrity Number (RIN) is not recommended for this application.

3 Grade each RNA sample based on the percentage of RNA in the sample >200 nucleotides, according to **Table 19**.

Table 19 Classification of FFPE RNA samples based on starting RNA size

Grade	DV200	Recommended input amount	Minimum input amount
Good FFPE RNA	>50%	200 ng	10 ng
Poor FFPE RNA	20% to 50%	200 ng	50 ng*
Inapplicable FFPE RNA	<20%	Not recommended for further processing	

* For optimal results, prepare libraries from poor-grade FFPE RNA samples using a minimum of 50 ng input RNA. Libraries may be prepared from 10–50 ng poor-grade FFPE RNA with potential negative impacts on yield or NGS performance.

4 Place 10 µL of each sample, containing 10–200 ng of FFPE total RNA in nuclease-free water, into wells of a PCR plate.

Poor-quality FFPE samples should contain at least 50 ng RNA.

Run the automated fragmentation protocol (both FFPE and intact RNA)

Intact total RNA samples (i.e., RNA prepared from fresh or fresh frozen samples or commercially-prepared reference RNA) require fragmentation prior to cDNA synthesis, while FFPE-derived RNA samples are already sufficiently fragmented and do not require further fragmentation. Importantly, however, FFPE RNA still needs to be run in the automated fragmentation protocol (**00a RNA_Frag_XT_HS2_ILM**) in order to mix the samples with the 2X Priming Buffer, which includes both fragmentation agents and primers needed for cDNA synthesis in the following steps. At the end of the automated fragmentation protocol, intact RNA samples are transferred to a thermal cycler to be fragmented at 94°C (see thermal cycling program in **Table 20**). FFPE RNA samples are not subjected to this thermal cycling program.

CAUTION Make sure to read the instructions in this section carefully as some steps differ depending on the RNA sample type (FFPE RNA or intact RNA).

Prepare the NGS Bravo for protocol 00a RNA_Frag_XT_HS2_ILM (both FFPE and intact RNA)

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

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

3 Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 21. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.

4 Place a red PCR plate insert at Bravo deck position 6.

Pre-program the thermal cycler (intact RNA only)

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

Table 20 Thermal cycler program for fragmentation of intact RNA samples*

Step	Temperature	Time
Step 1	94°C	4 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

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

NOTE When using the SureCycler 8800 thermal cycler, the heated lid may be left on (default setting) throughout the RNA library preparation incubation steps.

Prepare the sample plate for the fragmentation protocol (both FFPE and intact RNA)

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

Prepare the Fragmentation master mix source plate (both FFPE and intact RNA)

7 Prepare the **Agilent Deep Well** source plate for the run as indicated in **Table 21**. Add the indicated volume of 2X Priming Buffer to all wells of the indicated column of the Agilent Deep Well plate. Keep the 2X Priming Buffer on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 3**.

Table 21 lists the required volume of 2X Priming Buffer per well for 1 to 12 columns of samples. Make sure to use the volume appropriate for your run.

Table 21 Preparation of the Fragmentation master mix source plate for protocol 00a RNA_Frag_XT_HS2_ILM

Solution	Position on Source Plate	Volume (µL) of master mix added per Well of Agilent Deep Well Source Plate Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
2X Priming Buffer (tube with purple cap)	Column 1 (A1-H1)	19.8	31.8	43.8	55.8	67.8	79.8	91.7	103.8	115.9	128.0	140.1	152.2

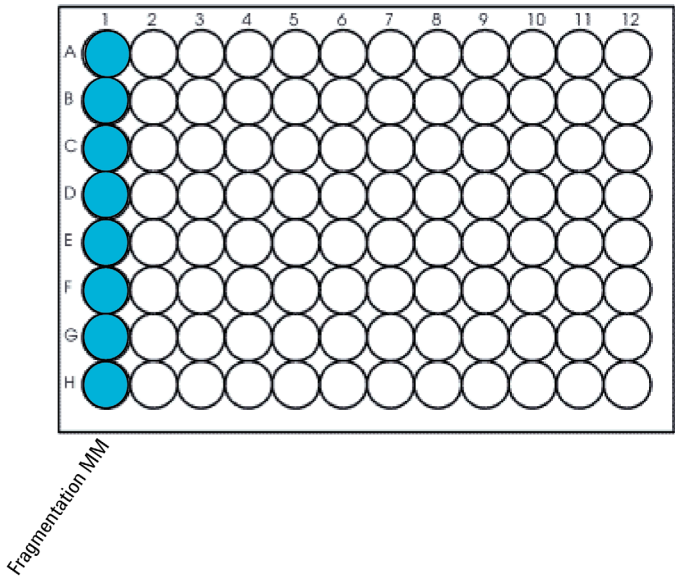


Figure 3 Configuration of the **Agilent Deep Well** source plate for protocol 00a RNA_Frag_XT_HS2_ILM

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

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

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

Load the NGS Bravo

10 Load the Bravo deck according to **Table 22**.

Table 22 Initial Bravo deck configuration for protocol 00a RNA_Frag_XT_HS2_ILM

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

Run VWorks protocol 00a RNA_Frag_XT_HS2_ILM (both FFPE and intact RNA)

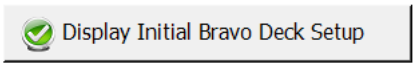
11 On the SureSelect setup form, under **Select protocol to execute**, select the **00a RNA_Frag_XT_HS2_ILM** protocol.

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

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

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

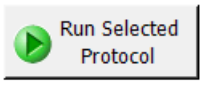
15 Click **Display Initial Bravo Deck Setup**.



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

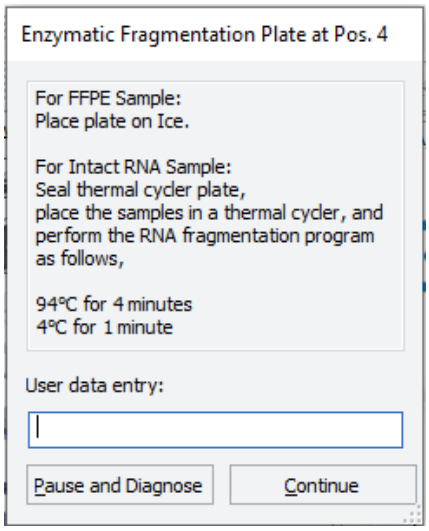
17 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **00a RNA_Frag_XT_HS2_ILM** protocol takes approximately 10 minutes. Once complete, the samples are ready for fragmentation (performed in the pre-programmed thermal cycler with intact RNA samples only). The samples are located in the PCR plate at position 4 of the Bravo deck.

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



20 Vortex the sealed plate at medium speed for 5 seconds, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

21 Proceed as needed for your RNA sample type.

- **Intact RNA**: Immediately place the sample plate in the thermal block. Close the lid and resume the thermal cycler program in **Table 20**. Once the thermal cycler program reaches the 4°C Hold step, transfer the sample plate from the thermal cycler to ice or a cold block. This plate is used as the RNA sample plate for first strand cDNA synthesis.
- **FFPE RNA**: Transfer the sample plate to ice or a cold block. This plate is used as the RNA sample plate for first strand cDNA synthesis.

22 From the Bravo deck, remove the Agilent Deep Well 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 **00b RNA_FirstStrand_XT_HS2_ILM** protocol as described in **"Prepare the First Strand cDNA master mix source plate"** on page 55.

23 Proceed immediately to **"Step 2. Synthesize first strand cDNA"** on page 54.

Step 2. Synthesize first strand cDNA

This step uses automation protocol **00b RNA_FirstStrand_XT_HS2_ILM**.

In this step, the NGS Bravo executes preparation of the sample plate containing the RNA samples and reagents for first strand cDNA synthesis. The plate is then transferred to the thermal cycler to run a cycling program that directs the synthesis of first strand cDNA.

Prepare the NGS Bravo for protocol 00b RNA_FirstStrand_XT_HS2_ILM

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
3. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 21. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
4. Place a red PCR plate insert at Bravo deck position 6.

Pre-program the thermal cycler for first strand cDNA synthesis

1. 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 first-strand cDNA synthesis*

Step	Temperature	Time
Step 1	25°C	10 minutes
Step 2	37°C	40 minutes
Step 3	4°C	Hold

* Use a reaction volume setting of 28 µL, if required for thermal cycler set up.

Prepare the First Strand cDNA master mix source plate

1 Prepare the **Agilent Deep Well** source plate for the run as indicated in **Table 24**. Add the indicated volume of First Strand Master Mix to all wells of the indicated column of the Agilent Deep Well plate. Keep the master mix on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 4**.

Table 24 lists the required volume of First Strand Master Mix per well for 1 to 12 columns of samples. Make sure to use the volume appropriate for your run.

CAUTION

The First Strand Master Mix used in this step is viscous. Mix thoroughly by vortexing at high speed for 5 seconds before removing an aliquot for use. Pipetting up and down is not sufficient to mix this reagent.

The First Strand Master Mix is provided with actinomycin-D already supplied in the mixture. Do not supplement with additional actinomycin-D.

Table 24 Preparation of the First Strand cDNA master mix source plate for protocol 00b RNA_FirstStrand_XT_HS2_ILM

Solution	Position on Source Plate	Volume (µL) of master mix added per Well of Agilent Deep Well Source Plate Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
First Strand Master Mix (amber tube with amber cap)	Column 2 (A2-H2)	16.8	27.0	37.2	47.4	57.6	67.8	78.0	88.3	98.5	108.8	119.1	129.4

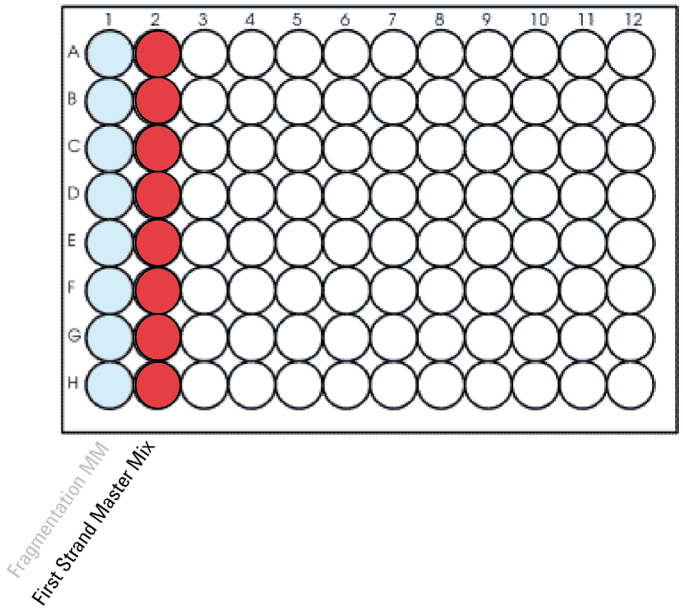


Figure 4 Configuration of the Agilent Deep Well source plate for protocol 00b RNA_FirstStrand_XT_HS2_ILM. The master mix dispensed during a previous protocol is shown in light shading.

2 Seal the 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.

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

Load the NGS Bravo

1 Load the Bravo deck according to **Table 22**.

Table 25 Initial Bravo deck configuration for protocol 00b RNA_FirstStrand_XT_HS2_ILM

Location	Content
2	New tip box
4	RNA sample plate (from **step 21** on **page 53**) seated in red insert (PCR plate type must be specified on setup form under Parameter 2)
5	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
8	Empty tip box
9	First Strand cDNA master mix source plate, unsealed

Run VWorks protocol 00b RNA_FirstStrand_XT_HS2_ILM

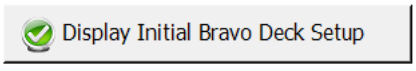
1 On the SureSelect setup form, under **Select protocol to execute**, select the **00b RNA_FirstStrand_XT_HS2_LM** protocol.

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

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

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

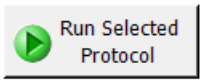
5 Click **Display Initial Bravo Deck Setup**.



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

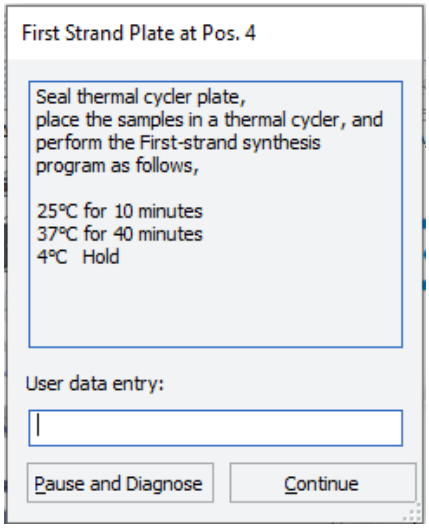
7 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **00b RNA_FirstStrand_XT_HS2_ILM** protocol takes approximately 10 minutes. Once complete, the samples are ready for first-strand cDNA synthesis (performed in the preprogrammed thermal cycler). The samples are located in the PCR plate at position 4 of the Bravo deck.

9 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.



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

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

12 From the Bravo deck, remove the Agilent Deep Well plate that was used as the First Strand cDNA Master Mix source plate from position 9 and set it aside. You will use this same plate again for the **00c RNA_SecondStrand_XT_HS2_ILM** protocol as described in **"Prepare the Second Strand master mix and master mix source plate"** on page 58.

13 Once the thermal cycler program in **Table 23** reaches the 4°C Hold step, transfer the first strand cDNA sample plate from the thermal cycler to ice or a cold block. Proceed immediately to **"Step 3. Synthesize second strand cDNA"** on page 58.

Step 3. Synthesize second strand cDNA

This step uses automation protocol **00c RNA_SecondStrand_XT_HS2_ILM**.

In this step, the first strand cDNA is used as a template to synthesize second strand cDNA.

Prepare the NGS Bravo for protocol 00c RNA_SecondStrand_XT_HS2_ILM

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
3. Pre-set the temperature of Bravo deck position 4 to 14°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
5. Place a red PCR plate insert at Bravo deck position 4.

Prepare the Second Strand master mix and master mix source plate

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

   Table 26 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

   a. Vortex the thawed vial of Second Strand Enzyme Mix for 5 seconds at high speed to ensure homogeneity.

CAUTION The Second Strand Enzyme Mix used in this step is viscous. Pipetting up and down is not sufficient to mix this reagent.

---

   b. Slowly pipette the Second Strand Enzyme Mix into a 1.5-mL Eppendorf tube or conical vial, ensuring that the full volume is dispensed.

   c. Add the Second Strand Oligo Mix. 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 26 Preparation of Second Strand master mix

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Second Strand Enzyme Mix (bottle)	25 µL	310.5	550.8	791.2	1031.5	1367.5	1610.1	1852.6	2095.2	2337.7	2580.3	2822.8	3065.4
Second Strand Oligo Mix (tube with yellow cap)	5 µL	62.1	110.2	158.2	206.3	273.5	322.0	370.5	419.0	467.5	516.1	564.6	613.1
Total Volume	**30 µL**	**372.8**	**661.0**	**949.4**	**1237.8**	**1641.0**	**1932.1**	**2223.2**	**2514.2**	**2805.3**	**3096.3**	**3387.4**	**3678.5**

2 Prepare the Agilent Deep Well source plate for the run as indicated in **Table 27**. Add the indicated volume of Second Strand master mix 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 27 lists the required volume of Second Strand master mix for 1 to 12 columns of samples. Make sure to use the volume appropriate for your run.

Table 27 Preparation of the Second Strand master mix source plate for protocol 00c RNA_SecondStrand_XT_HS2_ILM

Solution	Position on Source Plate	Volume (µL) of master mix added per Well of Agilent Deep Well Source Plate Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Second Strand Master Mix	Column 3 (A3-H3)	44.4	78.7	113.0	147.4	195.4	230.0	264.7	299.3	334.0	368.6	403.3	437.9

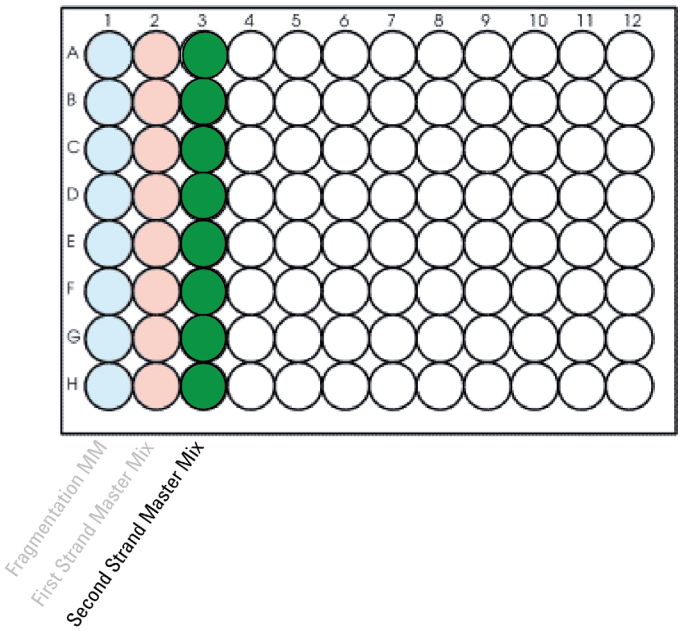


Figure 5 Configuration of the Agilent Deep Well source plate for protocol 00c RNA_SecondStrand_XT_HS2_ILM. The master mixes dispensed during previous protocols are shown in light shading.

3 Seal the 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. Keep the master mix source plate on ice.

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

Load the NGS Bravo

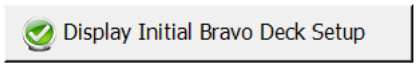
1 Load the Bravo deck according to **Table 28**.

Table 28 Initial Bravo deck configuration for protocol 00c RNA_SecondStrand_XT_HS2_ILM

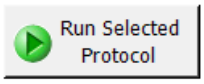
Location	Content
2	New tip box
4	First strand cDNA samples in PCR plate seated in red insert (PCR plate type must be specified on setup form under Parameter 2)
5	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
8	Empty tip box
9	Second Strand master mix source plate, unsealed

Run VWorks protocol SecondStrandcDNA_XT_HS2_RNA

1. On the SureSelect setup form, under **Select protocol to execute**, select the **00c RNA_SecondStrand_XT_HS2_ILM** protocol.
2. Under **Select labware for thermal cycling**, select the specific type of PCR plate used at position 4 of the Bravo deck.
3. Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.
4. Select the number of columns of samples to be processed.
5. Click **Display Initial Bravo Deck Setup**.



6. Verify that the Bravo deck has been set up as displayed on the right side of the form.
7. Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.
8. When verification is complete, click **Run Selected Protocol**.



Running the **00c RNA_SecondStrand_XT_HS2_ILM** protocol takes approximately 70 minutes. Once complete, the cDNA samples are ready for purification. The samples are located in the processing plate at position 4 of the Bravo deck. Transfer the cDNA sample plate from the Bravo deck to ice or a cold block.

From the Bravo deck, remove the Agilent Deep Well plate that was used as the Second Strand master mix source plate from position 6 and set it aside. You will use this same plate again for the **01 LibraryPrep_XT_HS2_LIM** protocol as described in **"Prepare the Library Prep master mix source plate"** on page 69.

Step 4. Purify cDNA using AMPure XP beads

This step uses automation protocol **00d Cleanup_cDNA_XT_HS2_ILM**.

In this step, the NGS Bravo performs the purification steps for the cDNA using AMPure XP beads. This step uses the aliquoted plate of AMPure XP beads that was prepared on **page 36**.

Prepare the NGS Bravo and reagent reservoirs for protocol 00d Cleanup_cDNA_XT_HS2_ILM

At the end of the automation protocol, you may retain the reservoirs for later use in the **04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM** or **04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM** protocol if you are running that protocol today.

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
3. Pre-set the temperature of Bravo deck position 6 to 45°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
4. Prepare an Agilent deep well reservoir containing 30 mL of nuclease-free water.
5. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.
6. Place a red PCR plate insert at Bravo deck position 4.

Load the NGS Bravo

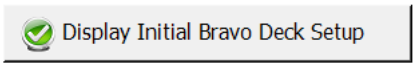
1. Load the Bravo deck according to **Table 29**.

Table 29 Initial Bravo deck configuration for protocol 00d Cleanup_cDNA_XT_HS2_ILM

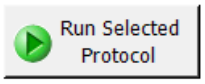
Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
3	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
4	cDNA samples in PCR plate seated in red insert (PCR plate type must be specified on setup form under Parameter 2)
5	Aliquotted AMPure XP beads in Agilent Deep Well plate from **page 36** (105 µL of beads/well)
8	Empty tip box
9	70% ethanol reservoir from **step 5**, above

Run VWorks protocol 00d Cleanup_cDNA_XT_HS2_ILM

1. On the SureSelect setup form, under **Select Protocol**, select the **00d Cleanup_cDNA_XT_HS2_ILM** protocol.
2. Under **Select labware for thermal cycling**, select the specific type of PCR plate used at position 4 of the Bravo deck.
3. Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.
4. Select the number of columns of samples to be processed.
5. Click **Display Initial Bravo Deck Setup**.



6. Verify that the Bravo deck has been set up as displayed on the right side of the form.
7. Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.
8. When verification is complete, click **Run Selected Protocol**.



Running the **00d Cleanup_cDNA_XT_HS2_ILM** protocol takes approximately 40 minutes. An operator must be present during the run to complete tip box replacement and to transfer the water reservoir to the Bravo deck, when directed by the VWorks prompt shown below.

Once the protocol is complete, the purified cDNA samples are located in the processing plate at position 3 of the Bravo deck. [[seal plate and keep on ice if they are continuing to next step?]]

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

5 Library Preparation

This chapter describes the steps for the automated preparation of cDNA NGS libraries for sequencing using the Illumina paired-read platform. For each sample to be sequenced, an individual dual-indexed and molecular-barcoded library is prepared. For an overview of the SureSelect XT HS2 target enrichment workflow, see **Figure 2** on page 28.

The protocol requires 10 ng to 200 ng of input cDNA. 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 to 50 ng) or when detecting very low allele frequency variants using small probe designs.

Step 1. Prepare adaptor-ligated libraries

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

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

This step uses the components listed in **Table 30**. Thaw and mix each component as directed in **Table 30** 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 30 Reagents thawed before use in protocol LibraryPrep_XT_HS2_ILM

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
End Repair-A Tailing Buffer (bottle)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	**page 67**
Ligation Buffer (bottle)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	**page 68**
End Repair-A Tailing Enzyme Mix (tube with orange cap)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Place on ice just before use	Inversion	**page 67**
T4 DNA Ligase (tube with blue cap)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Place on ice just before use	Inversion	**page 68**
XT HS2 RNA Adaptor Oligo Mix (tube with green cap)	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	**page 68**

Prepare the NGS Bravo for protocol 01 LibraryPrep_XT_HS2_ILM

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Pre-set the temperature of Bravo deck position 4 to 79°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
3. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
4. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
5. Place a red PCR plate insert at Bravo deck position 4.

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 31** and using the liquid handling steps specified below.

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

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

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

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

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

Table 31 Preparation of End Repair/dA-Tailing master mix

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
End Repair-A Tailing Buffer (bottle)	16 µL	204	340	476	612	748	884	1042.7	1201.3	1360	1518.7	1677.3	1836
End Repair-A Tailing Enzyme Mix (tube with orange cap)	4 µL	51	85	119	153	187	221	260.7	300.3	340	379.7	419.3	459
Total Volume	**20 µL**	**255**	**425**	**595**	**765**	**935**	**1105**	**1303.4**	**1501.6**	**1700**	**1894.4**	**2096.6**	**2295**

Prepare the Ligation master mix

1 Prepare the appropriate volume of Ligation master mix, using volumes listed in **Table 32** and using the liquid handling steps specified below.

Table 32 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

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

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

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

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

Table 32 Preparation of Ligation master mix

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Ligation Buffer (bottle)	23 µL	293.3	488.8	684.3	879.8	1075.3	1270.8	1515.1	1759.5	2003.9	2248.3	2492.6	2737
T4 DNA Ligase (tube with blue cap)	2 µL	25.5	42.5	59.5	76.5	93.5	110.5	127.5	153.0	174.3	195.5	216.8	238
Total Volume	**25 µL**	**318.8**	**531.3**	**743.8**	**956.3**	**1168.8**	**1381.3**	**1642.6**	**1912.5**	**2178.2**	**2443.8**	**2709.4**	**2975**

Prepare the Adaptor Oligo Mix

1 Prepare the appropriate volume of Adaptor Oligo Mix dilution, according to **Table 33**. Mix well using a vortex mixer and keep on ice.

Table 33 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 33 Preparation of Adaptor Oligo Mix dilution

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	2.5 µL	42.5	63.8	85.0	106.3	127.5	143.5	170.0	191.3	212.5	233.8	255.0	276.3
XT HS2 RNA Adaptor Oligo Mix* (tube with green cap)	5 µL	85.0	127.5	170.0	212.5	255.0	287.0	340.0	382.5	425.0	467.5	510.0	552.5
Total Volume	**7.5 µL**	**127.5**	**191.3**	**255.0**	**318.8**	**382.5**	**430.5**	**510.0**	**573.8**	**637.5**	**701.3**	**765.0**	**828.8**

* You can substitute the XT HS2 RNA Adaptor Oligo Mix with the XT HS2 DNA Adaptor Oligo Mix from the SureSelect XT HS2 DNA Library Preparation Kit for ILM. If you do so, prepare a 1:5 dilution of the XT HS2 DNA Adaptor Oligo Mix using low TE buffer before adding the Adaptor Oligo Mix to the master mix.

Prepare the Library Prep master mix source plate

1 Prepare the **Agilent Deep Well** master mix source plate containing the mixtures prepared above (**Prepare the DNA End-Repair/dA-Tailing master mix**, **Prepare the Ligation master mix**, and **Prepare the Adaptor Oligo Mix**). Add the volumes indicated in **Table 34** 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 6**.

Table 34 lists the required volume of each master mix per well for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 34 Preparation of the master mix source plate for protocol 01 LibraryPrep_XT_HS2_ILM

Master Mix Solution	Position on Source Plate	Volume (µL) of master mix added per Well of Agilent Deep Well Source Plate Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
End Repair-dA Tailing master mix	Column 4 (A4-H4)	31.0	52.0	73.0	94.0	115.0	136.0	158.8	182.0	205.8	230.0	254.8	280.0
Ligation master mix	Column 5 (A5-H5)	36.0	62.0	88.0	114.0	140.0	166.0	195.7	226.5	258.3	291.1	325.1	360.0
Adaptor Oligo Mix dilution	Column 6 (A6-H6)	15.0	22.5	30.0	37.5	45.0	52.5	60.6	68.8	76.9	85.0	93.1	101.3

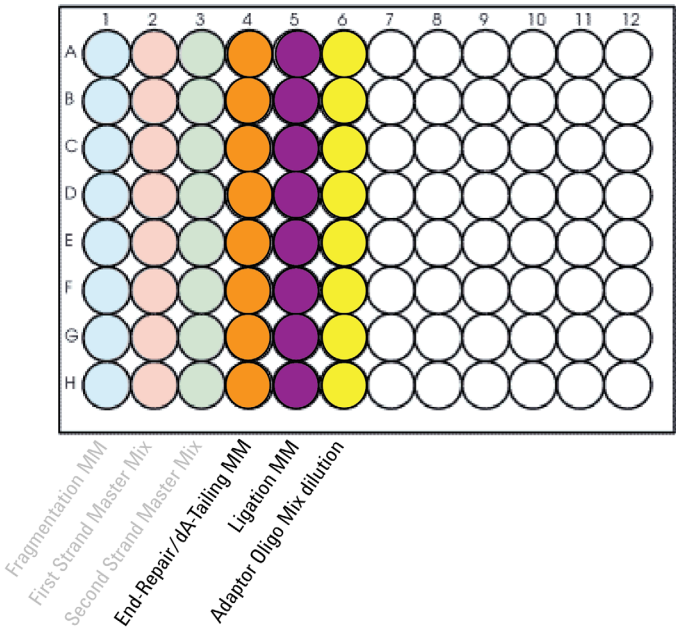


Figure 6 Configuration of the **Agilent Deep Well** master mix source plate for protocol 01 LibraryPrep_XT_HS2_ILM. The master mixes dispensed during previous protocols are shown in light shading.

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

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

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

Load the NGS Bravo

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

Table 35 Initial Bravo deck configuration for protocol 01 LibraryPrep_XT_HS2_ILM

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

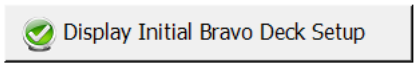
Run VWorks protocol 01 LibraryPrep_XT_HS2_ILM

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

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

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

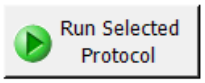
4 Click **Display Initial Bravo Deck Setup**.



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

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

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



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

Step 2. Purify adaptor-ligated libraries using AMPure XP beads

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

In this step, the NGS Bravo purifies the adaptor-ligated cDNA libraries using AMPure XP beads. This step uses the aliquoted plate of AMPure XP beads that was prepared on **page 38**.

Prepare the NGS Bravo and reagent reservoirs for protocol 02 Cleanup_LibPrep_XT_HS2_ILM

You can reuse the reservoirs that were used in the **00d Cleanup_cDNA_XT_HS2_RNA** protocol or use fresh reservoirs.

At the end of the automation protocol, you may retain the reservoirs for later use in the **04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM** or **04b Cleanup_Pre-CapPCR_SP_MP_HS2_ILM** protocol if you are running that protocol today.

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Pre-set the temperature of Bravo deck position 4 to 45°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
3. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
4. Prepare an Agilent deep well reservoir containing 30 mL of nuclease-free water.
5. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.

Load the NGS Bravo

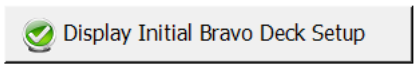
1. Load the Bravo deck according to **Table 36**.

Table 36 Initial Bravo deck configuration for protocol 02 Cleanup_LibPrep_XT_HS2_ILM

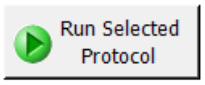
Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
5	Aliquotted AMPure XP beads in Agilent Deep Well plate from **page 38** (80 µL of beads/well)
6	Processing plate (Eppendorf twin.tec or Armadillo plate) containing adaptor-ligated libraries
8	Empty tip box
9	70% ethanol reservoir from **step 5**, above

Run VWorks protocol 02 Cleanup_LibPrep_XT_HS2_ILM

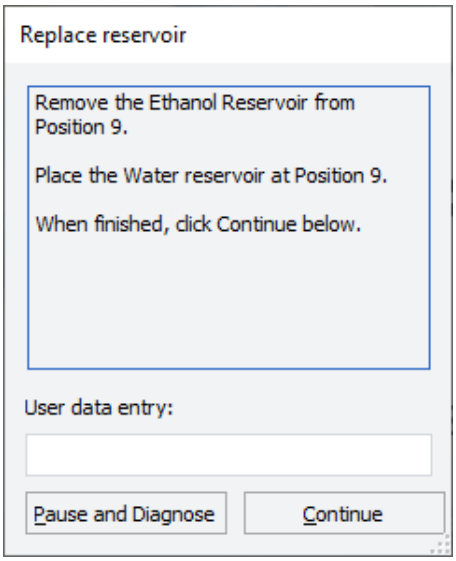
1. On the SureSelect setup form, under **Select Protocol**, select the **02 Cleanup_LibPrep_XT_HS2_ILM** protocol.
2. Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.
3. Select the number of columns of samples to be processed.
4. Click **Display Initial Bravo Deck Setup**.



5. Verify that the Bravo deck has been set up as displayed on the right side of the form.
6. Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.
7. When verification is complete, click **Run Selected Protocol**.



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



Once the protocol is complete, the purified cDNA samples are located in the Agilent Deep Well 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 3. Amplify adaptor-ligated libraries

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

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

This step uses the components listed in **Table 37**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 37 Reagents for pre-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (tube with red cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Pipette up and down 15–20 times	**page 76**
5× Herculase II Reaction Buffer (tube with clear cap)	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	**page 76**
SureSelect XT HS2 Index Primer Pairs	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR),* –20°C	Vortexing	**page 75**

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

CAUTION

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

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

Prepare the NGS Bravo for protocol 03 Pre-CapPCR_XT_HS2_ILM

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

Pre-program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 38**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the automation run.

Table 38 Pre-Capture PCR Thermal Cycler Program*

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10 to 14 (see **Table 39** for RNA input-based cycle number recommendations)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

* Use a reaction volume setting of 50 µL, if required for thermal cycler set up.

Table 39 Pre-capture PCR cycle number recommendations

Quality of Input DNA	Quantity of Input DNA	Cycles
Intact RNA	100 to 200 ng	10 cycles
	50 ng	11 cycles
	10 ng	12 cycles
Good quality FFPE RNA (DV200>50%)	100 to 200 ng	12 cycles
	50 ng	13 cycles
	10 ng	14 cycles
Poor quality FFPE RNA (DV200 20% to 50%)	100 to 200 ng	13 cycles
	50 ng	14 cycles

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 1** on **page 77** for the **03 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 40**. Vortex at medium speed for 15–20 seconds and keep on ice.

Table 40 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 40 Preparation of Pre-Capture PCR Master Mix

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
5× Herculase II Buffer with dNTPs (tube with clear cap)	10 µL	170.0	255.0	340.0	425.0	510.0	574.0	656.0	738.0	820.0	902.0	984.0	1066
Herculase II Fusion DNA Polymerase (tube with red cap)	1 µL	17.0	25.5	34.0	42.5	51.0	57.4	65.6	73.8	82.0	90.2	98.4	106.6
Total Volume	**11 µL**	**187.0**	**280.5**	**374.0**	**467.5**	**561.0**	**631.4**	**721.6**	**811.8**	**902.0**	**992.2**	**1082.4**	**1172.6**

2 Using the selected processing plate (Eppendorf twin.tec or Armadillo plate) as the master mix source plate, add the volume of PCR master mix indicated in **Table 41** to all wells of column 1 of the master mix source plate. The final configuration of the master mix source plate is shown in **Figure 7**.

Table 41 lists the required master mix volume per well for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 41 Preparation of the Pre-Capture PCR master mix source plate for protocol Pre-CapPCR_XT_HS2_ILM

Master Mix Solution	Position on Source Plate	Volume (µL) of master mix added per Well of Armadillo Source Plate Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Pre-Capture PCR Master Mix	Column 1 (A1-H1)	22	33	44	55	66	77	88	99	110	121	132	143

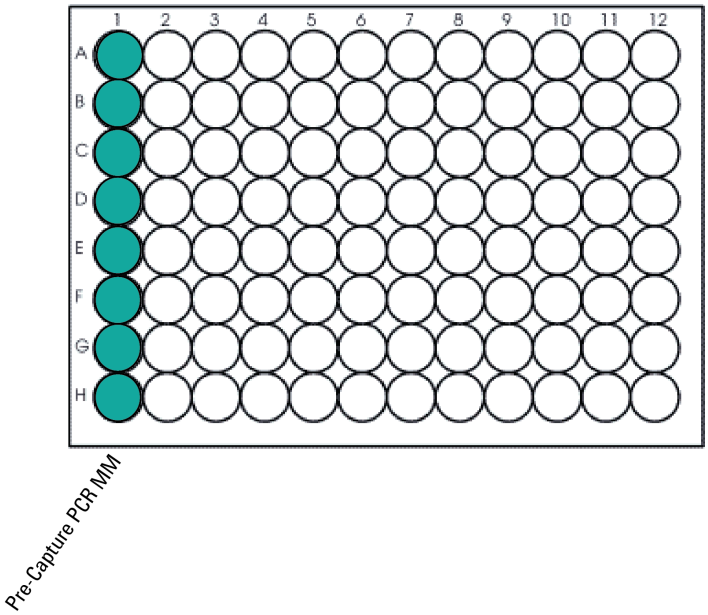


Figure 7 Configuration of the master mix source plate (Eppendorf twin.tec or Armadillo plate) for protocol 03 Pre-CapPCR_XT_HS2_ILM

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.

NOTE

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

Load the NGS Bravo

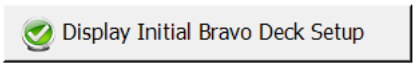
1 Load the Bravo deck according to **Table 42**.

Table 42 Initial Bravo deck configuration for protocol 03 Pre-CapPCR_XT_HS2_ILM

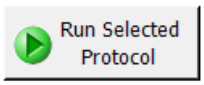
Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
6	SureSelect XT HS2 Index Primer Pairs for ILM in PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
7	Purified, adaptor-ligated cDNA samples in Agilent Deep Well plate
8	Empty tip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

Run VWorks protocol 03 Pre-CapPCR_XT_HS2_ILM

1. On the SureSelect setup form, under **Select protocol to execute**, select the **03 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. Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.
4. Select the number of columns of samples to be processed.
5. Click **Display Initial Bravo Deck Setup**.

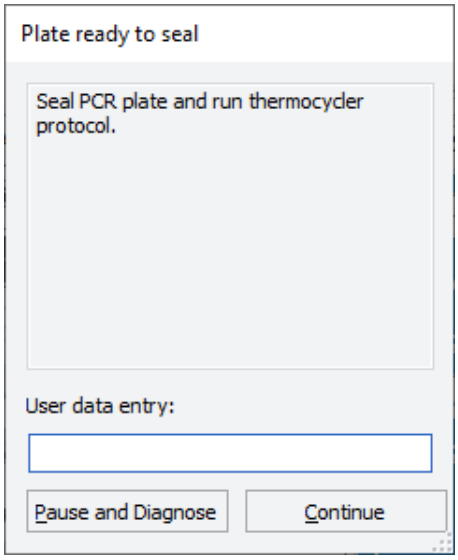


6. Verify that the Bravo deck has been set up as displayed on the right side of the form.
7. Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.
8. When verification is complete, click **Run Selected Protocol**.



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

9. 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.



10. Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.
11. 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 38**. Once the cycler has reached 98°C, immediately place the sample plate in the thermal block and close the lid.

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

Retain the master mix source plate (Eppendorf twin.tec or Armadillo plate) containing the Pre-Capture PCR master mix located at position 9 of the Bravo deck for later use in the **05 TS_D1000** protocol (see **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape"** on page 83).

Step 4. Purify amplified cDNA using AMPure XP beads

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

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

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

Prepare the NGS Bravo and reagent reservoirs for the pre-capture PCR cleanup protocol

You can reuse the reservoirs that were used in the **00d Cleanup_cDNA_XT_HS2_ILM** protocol or use fresh reservoirs.

At the end of the automation protocol, you may retain the reservoirs for later use in either the **06a Aliquot_Libraries** or **06b Aliquot_Water** protocol if you are running one of those protocols today.

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Pre-set the temperature of Bravo deck position 4 to 45°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
3. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
4. Prepare an Agilent deep well reservoir containing 30 mL of nuclease-free water.
5. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.
6. Place a red PCR plate insert at Bravo deck position 6.

Load the NGS Bravo

1 Load the Bravo deck according to **Table 43**.

Table 43 Initial Bravo deck configuration for the 04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM or 04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
3	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
5	Aliquotted AMPure XP beads in Agilent Deep Well plate from **page 40** (50 µL of beads/well)
6	Amplified cDNA libraries in unsealed PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
8	Empty tip box
9	70% ethanol reservoir from **step 5** on **page 80**

Run the appropriate Cleanup_Pre-CapPCR protocol

1 On the SureSelect setup form, under **Select protocol to execute**, select one of the Cleanup_Pre-CapPCR protocols based on your workflow of choice.

- If you are using the post-capture pooling workflow (i.e., pooling samples after hybridization with the Probe) then select the protocol **04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM**.
- If you are using the pre-capture pooling workflow (i.e., pooling samples prior to hybridization with the Probe) then select the protocol **04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM**.

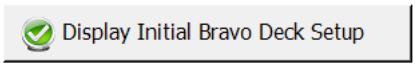
The 04a and 04b protocols use different elution volumes. Selecting the correct option is important for the downstream hybridization protocol.

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

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

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

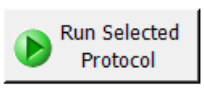
5 Click **Display Initial Bravo Deck Setup**.



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

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

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



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

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

Step 5. Assess Library cDNA quantity and quality

Sample analysis can be completed using one of two options.

- Option 1: Prepare the analytical assay plate using automation (protocol **05 TS_D1000**) and perform analysis on Agilent 4200 TapeStation. See **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape"** on page 83.
- 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 87.

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

This section describes the use of automation protocol **05 TS_D1000** to prepare the D1000 assay sample plate by combining 2 µL of each cDNA 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 ScreenTape Assay for TapeStation Systems Quick Guide.

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

Prepare the NGS Bravo and Sample Buffer source plate for protocol 05 TS_D1000

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Turn off the ThermoCube device (see **page 22**) to restore position 9 of the Bravo deck to room temperature.
3. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Using the same processing source plate (Eppendorf twin.tec or Armadillo plate) that was used in the **03 Pre-CapPCR_XT_HS2_ILM** protocol, prepare the Sample Buffer source plate at room temperature. Add the volume of D1000 Sample Buffer indicated in **Table 44** to each well of column 2 of the plate.

   Make sure to add the D1000 Sample Buffer to column 2 of the source plate.

Table 44 Preparation of the Sample Buffer source plate for protocol 05 TS_D1000

Solution	Position on Source Plate	Volume (µL) of Sample Buffer added per Well of Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
D1000 Sample Buffer	Column 2 (A2-H2)	11.0	17.0	23.0	29.0	35.0	41.0	47.0	53.0	59.0	65.0	71.0	77.0

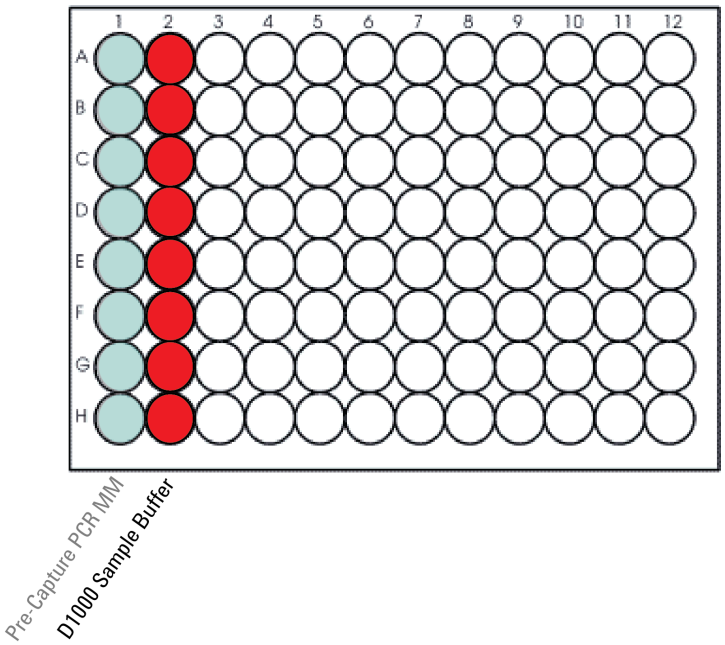


Figure 8 Configuration of the processing source plate for protocol **05 TS_D1000**. The master mix dispensed during a previous protocol is shown in light shading.

Load the NGS Bravo

5 Load the Bravo deck according to **Table 45**.

Table 45 Initial Bravo deck configuration for TS_D1000 protocol

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

CAUTION

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

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

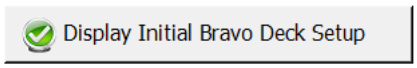
Run VWorks protocol 05 TS_D1000

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

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

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

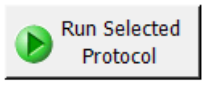
9 Click **Display Initial Bravo Deck Setup**.



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

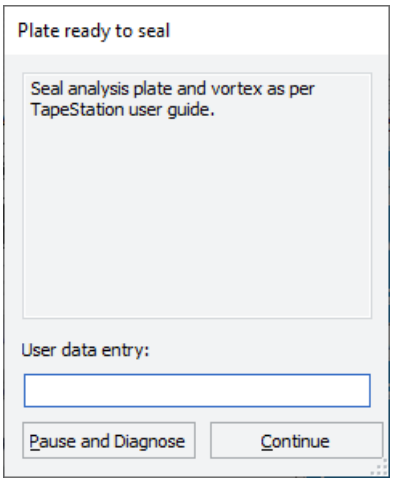
11 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



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

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



14 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 92**.

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 46** for guidelines). Sample electropherograms are shown in **Figure 9** (library prepared from high-quality RNA) and **Figure 10** (library prepared from typical FFPE RNA).

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 171** for additional considerations.

Table 46 Pre-capture library qualification guidelines

Input RNA type	Expected library DNA fragment size peak position	NGS read lengths supported
High quality RNA or FFPE RNA	200 to 700 bp	2 × 100 reads or 2 × 150 reads

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

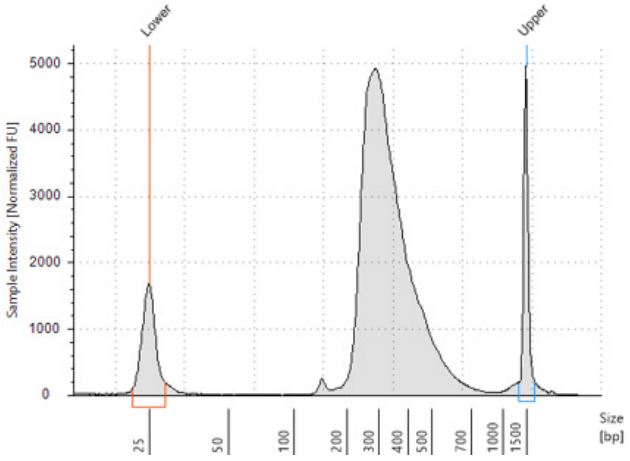


Figure 9 Pre-capture library prepared from high-quality RNA sample (Human Reference Total RNA) analyzed using a D1000 ScreenTape assay

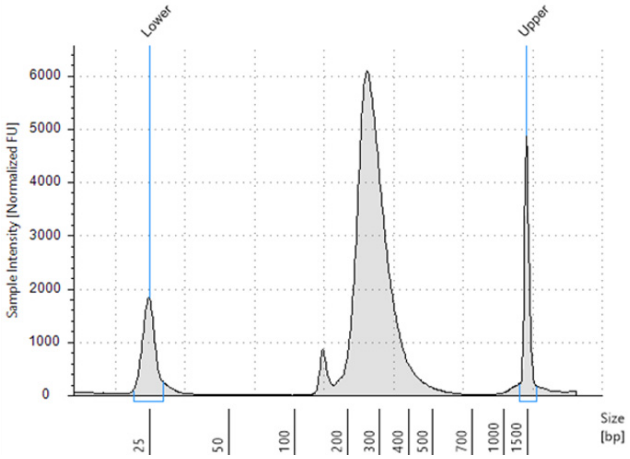


Figure 10 Pre-capture library prepared from a typical FFPE RNA sample analyzed using a D1000 ScreenTape assay

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 9** and **Figure 10**). Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 46** for guidelines). **Table 47** includes links to assay instruction.

Table 47 Pre-capture library analysis options

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

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

6 Hybridization

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

The first step is to prepare the cDNA 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 cDNA for Single-Plex Hybridization"** on page 90.
- If you are pooling samples prior to hybridization to the Probe, then follow the steps in **"Step 1, Option 2. Prepare cDNA for Multi-Plex Hybridization"** on page 93.

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

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

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

Follow the steps in this section if you are using the post-capture pooling workflow. If you are using the pre-capture pooling workflow, see **"Step 1, Option 2. Prepare cDNA for Multi-Plex Hybridization"** on page 93.

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

The hybridization reaction requires 200 ng of prepared cDNA in a volume of 12 µL. Use the maximum amount of prepared cDNA available within this range.

Calculate sample volumes needed for hybridization

The automation protocol Aliquot_Libraries is used to prepare a new sample plate containing the appropriate quantity of each cDNA sample for hybridization. It prepares 400 ng of cDNA sample in a volume of 24 µL, which is enough for two hybridization reactions.

1 Using the cDNA concentration for each sample determined on **page 83** to **page 87**, calculate the volume of each sample to be used for hybridization using the formula below:

   Volume (µL) = 400 ng/concentration (ng/µL)

If the concentration of any sample is not sufficient to allow use of the recommended 400 ng of cDNA, use the full remaining volume of cDNA sample (approximately 10 to 12 µL, containing at least 200 ng) for the hybridization step.

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

Prepare .csv file for sample normalization and aliquoting

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 cDNA 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 cDNA sample to be used in the hybridization step (see **"Calculate sample volumes needed for hybridization"** 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 A\XT_HS2_RNA_ILM_v.Ax.x.x\Aliquot Input File Templates\Aliquot_Libraries_Template.csv** (where x.x.x is the version number).

The Aliquot_Libraries_template.csv file may be copied and used as a template for creating the .csv files for each **06a 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 A\XT_HS2_RNA_ILM_v.Ax.x.x\Aliquot Input File Templates**.

Prepare the NGS Bravo and reagent reservoir for protocol 06a Aliquot_Libraries

You can reuse the reservoir that was used in the **Cleanup_Pre-CapPCR** protocol (04a or 04b) or use a fresh reservoir.

At the end of the automation protocol, you may retain the reservoir for later use in the **09 SSELWash_XT_HS2_ILM** protocol if you are running that protocol today.

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

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

3 Prepare an Agilent deep well reservoir containing 30 mL of nuclease-free water.

Load the NGS Bravo

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

Table 48 Initial Bravo deck configuration for protocol 06a Aliquot_Libraries

Location	Content
2	Nuclease-free water reservoir from **step 3**
5	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
6	Empty tip box
8	New, unused tip box*
9	Prepped library cDNA in destination plate (Eppendorf twin.tec or Armadillo plate)

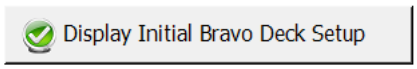
* The **06a Aliquot_Libraries** protocol does not use the Current Tip State indicator. The tip box at position 8 must be new and full of unused tips.

Run VWorks protocol 06a Aliquot_Libraries

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

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

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



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

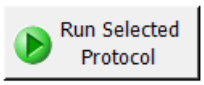
5 Upload the .csv file created in **step 1** through **step 3**.

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

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

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

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



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

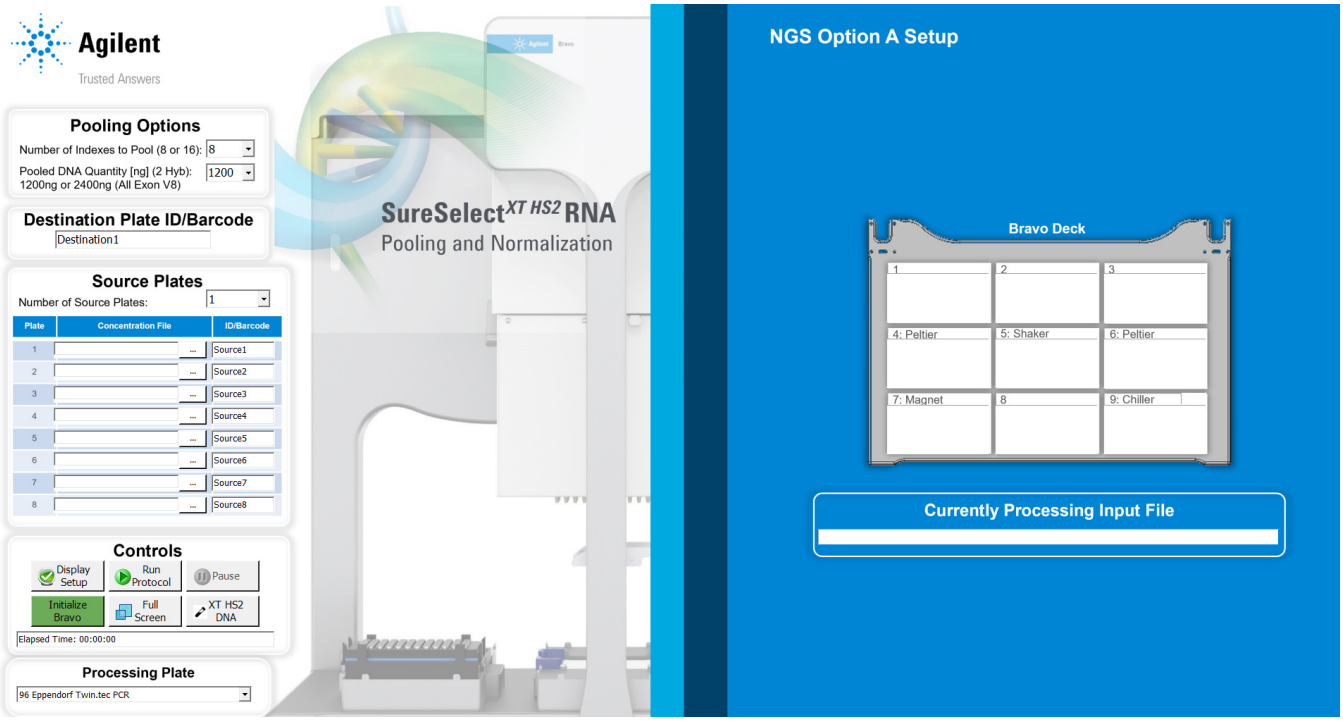
7 Remove the sample plate from the Bravo deck. Proceed directly to **"Step 2. Hybridize the cDNA library or library pool and probe"** on page 103.

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

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

- The **PreCapture_Pooling** automation protocol, which is set up using the XT_HS2_Pooling VWorks form (shown below), pools the prepped indexed cDNA samples. The form is accessible from within the XT_HS2_ILM form.
- The **06b Aliquot_Water** automation protocol adds enough water to each cDNA library pool to bring the volume to 100 µL.
- Then, **06c PoolingConcentration_XT_HS2_ILM** automation protocol uses AMPure XP beads (prepared on **page 42**) to purify the cDNA library pools, eluting the cDNA in a volume of 24 µL.

Follow the steps in this section if you are using the pre-capture pooling workflow. If you are using the post-capture pooling workflow, see **"Step 1, Option 1. Prepare cDNA for Single-Plex Hybridization"** on page 90.



Plan pooling run parameters

The hybridization reaction requires 1200 ng of indexed cDNA (or 2400 ng of indexed cDNA if using one of the SureSelect XT HS PreCap Human All Exon V8 Probes). The indexed cDNA is made up of a pool that contains equal amounts of 8 or 16 individual libraries. See **Table 49** for the recommended pool composition based on your SureSelect or ClearSeq Probe.

Table 49 Pre-capture pooling of indexed cDNA libraries

Probe	Amount of total cDNA per pool (Amount of cDNA pool per hybridization reaction)*	Number of indexed cDNA libraries per pool	Amount of each indexed cDNA library in pool	Maximum cDNA concentration for pool
SureSelect XT HS PreCap Human All Exon V8 Probe	2400 ng (1200 ng/hybridization)	8	300 ng	150 ng/µL
SureSelect XT HS PreCap Human All Exon V8+UTR Probe	2400 ng (1200 ng/hybridization)	8	300 ng	150 ng/µL
SureSelect Custom Probe	1200 ng (600 ng/hybridization)	16	75 ng	37.5 ng/µL
ClearSeq Comprehensive Cancer	1200 ng (600 ng/hybridization)	16	75 ng	37.5 ng/µL
SureSelect Human or Mouse All-Exon	1200 ng (600 ng/hybridization)	8	150 ng	75 ng/µL
SureSelect Clinical Research Exome	1200 ng (600 ng/hybridization)	8	150 ng	75 ng/µL
SureSelect Focused Exome	1200 ng (600 ng/hybridization)	8	150 ng	75 ng/µL
ClearSeq Inherited Disease	1200 ng (600 ng/hybridization)	8	150 ng	75 ng/µL

* Where possible, indexed cDNA pools are prepared containing a total cDNA amount that is enough for two hybridization reactions, i.e., 2400 ng for hybridizations with one of the SureSelect XT HS PreCap Human All Exon V8 Probes (V8 or V8+UTR) and 1200 ng for all other probes. For some indexed cDNA pools, the initial library pool will contain enough total cDNA for more than two hybridization reactions.

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

Accurate normalization of pools requires a minimum pipetting volume of 2 µL for each sample. Maximum cDNA concentration values for a pool containing >2 µL of each sample are shown in **Table 49**, above. When higher-concentration cDNA samples are included in the pooling run, the cDNA pool amount must be adjusted as described below.

- Check the cDNA concentration of each sample in the set of source plates to be pooled to a single destination plate to determine the appropriate amount of cDNA per pool.
  - If all samples contain cDNA at concentrations below the maximum cDNA concentration shown in **Table 49**, then prepare 1200 ng cDNA pools (or a 2400 ng pool if using one of the SureSelect XT HS PreCap Human All Exon V8 Probes).
  - If at least one of the samples is above the maximum cDNA concentration shown in **Table 49**, then you need to calculate the appropriate cDNA pool amount. First, identify the most concentrated cDNA sample and calculate the amount of cDNA contained in 2 µL of that sample. This becomes the amount of each cDNA sample used for pooling in the run. For example, if the highest cDNA sample concentration is 200 ng/µL, then the final cDNA pool will contain 400 ng of each indexed cDNA. Next, determine the total amount of cDNA 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 cDNA.

Plan destination indexed cDNA pool sample plate configuration

The indexed cDNA 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 cDNA 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 cDNA library pools to be hybridized to a particular Probe are positioned in appropriate rows. When preparing for the **07 Hyb_XT_HS2_ILM** protocol, place samples to be enriched using the same Probe in the same row.
- Each 96-reaction library preparation run produces 6 or 12 cDNA pools. For greatest efficiency of reagent use, cDNA pools from multiple library preparation runs may be placed on the same destination plate for hybridization.

Prepare .csv files for pooling and normalization

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

See **Figure 12** for required .csv file content. Pooling and normalization .csv file templates are provided in the following directory: **C:\VWorks Workspace\NGS Option A\XT_HS2_ILM_v.Ax.x.x\Pooling and Normalization Templates**.

1. Select the appropriate set of templates from the directory based on the intended pool composition (8 or 16 prepared samples) and on the number of source plates to be consolidated in the run to prepare the single destination plate containing the hybridization samples. For example, for 8-library pools, use the template Pool8_01_SourcePlate.csv for the first cDNA source plate, continuing with additional Pool8_0X_SourcePlate.csv files for additional cDNA source plates.
2. Copy and rename the appropriate set of .csv file templates for the run. Make sure to retain the header text used in the template files, without introducing spaces or other new characters.

   If processing a partial plate of prepped cDNA 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

3 In each .csv file, edit the information for each cDNA sample (well ID) as follows:

- In the **PreCap Amplified pond concentrations** field, enter the concentration (in ng/µL) determined on **page 83** for each indexed cDNA sample.
- In the **Target WellID** field, enter the well position of the pool in which the indexed cDNA sample should be included for the destination plate. See the guidelines on **page 95** for hybridization sample pool placement considerations.

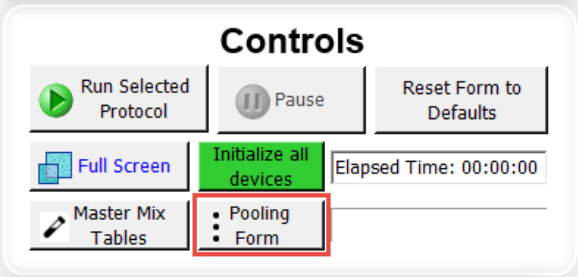
Set up and run the PreCapture_Pooling automation protocol

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

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

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

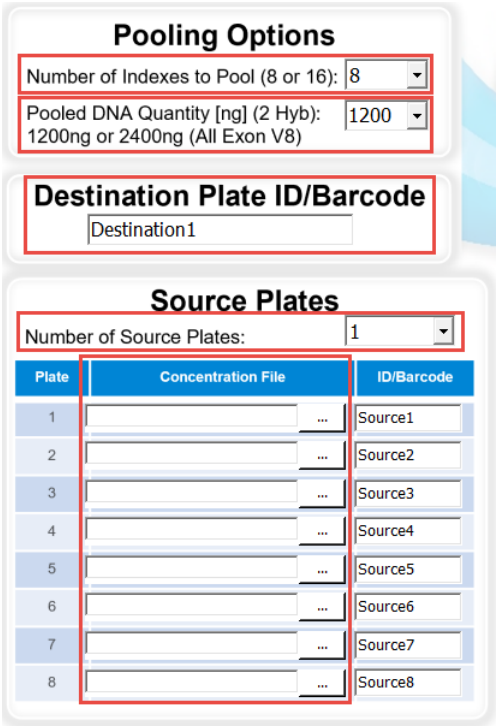
- Double-click the shortcut on your desktop for the XT2_HS2_Pooling VWorks Form.
- In the directory **C:\VWorks Workspace\NGS Option A\XT_HS2_ILM_v.Ax.x.x\Forms** (where x.x.x is the version number) open the file **XT_HS2_Pooling_v.Ax.x.x.VWForm**.
- From the XT_HS2_ILM VWorks Form, under **Controls**,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



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

- Under **Pooling Options**, from **Number of Indexes to Pool** menu, select 8 or 16 (see **Table 49** on page 94 for guidelines).

- Under **Pooling Options**, from **Pooled DNA Quantity** menu, enter the required total amount of cDNA in the pool. For the SureSelect XT HS PreCap Human All Exon V8 Probes, the required amount is 2400 ng. For all other Probes, the typical amount is 1200 ng. These amounts are sufficient for two hybridization reactions. See **page 93** for guidelines.
- Under **Destination Plate ID/Barcode**, enter the name or barcode of the destination plate into the field provided.
- Under **Source Plates**, from **Number of Source Plates** menu, select the number of indexed cDNA source plates to be provided for sample pooling. If >8 plates will be used to create a single hybridization sample plate, run the pooling and normalization protocol in sets of 8 source plates.
- In the table under **Source Plates**, in **Concentration File** field, use the browse button to specify the location of each .csv file that provides sample position and concentration data for each plate.

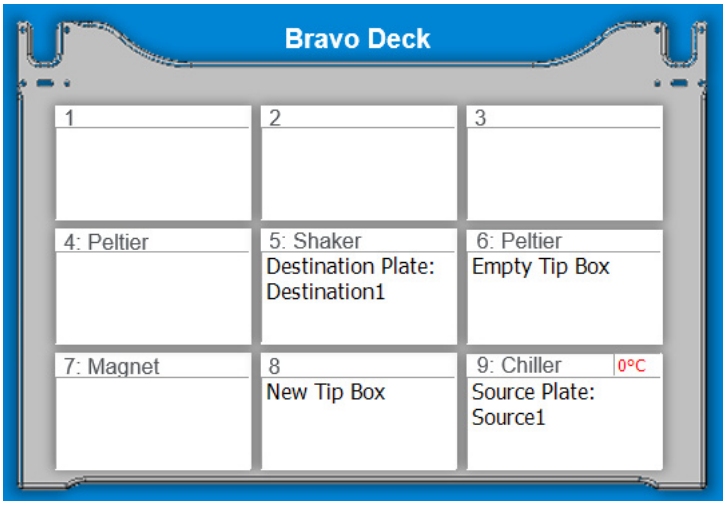


5 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

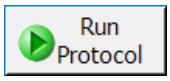
6 When finished entering run parameters in the Form, click **Display Setup**.

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

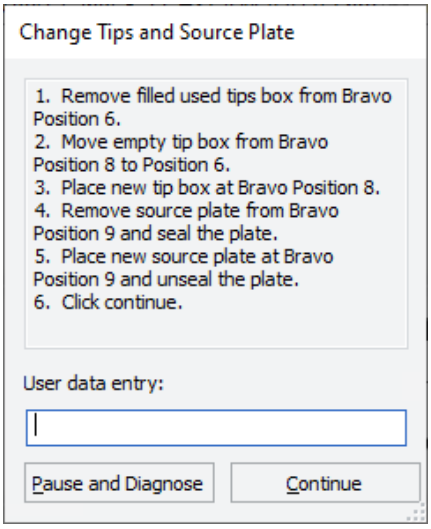
- Load the first indexed cDNA source plate on Bravo deck position 9. (If you are processing more than one source plate, you are prompted to load the subsequent source plates during the **PreCapture_Pooling** protocol.)
- Load an empty destination plate (Eppendorf twin.tec or Armadillo plate, as indicated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



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



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

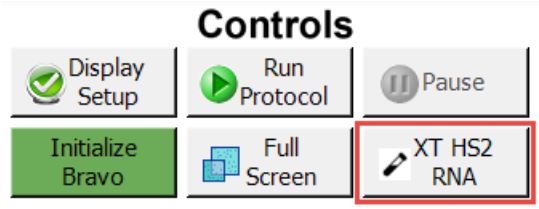


Running the **PreCapture_Pooling** protocol takes approximately one hour per indexed cDNA source plate. Once complete, the destination sample plate, containing indexed cDNA pools, is located at position 5 of the Bravo deck.

9 Remove the destination plate from Bravo deck position 5.

10 Seal the destination plate containing the indexed cDNA pool samples using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep on ice.

11 In the XT_HS2_Pooling VWorks form, under **Controls**, click **XT HS2 RNA** to return to the RNA_XT_HS2_ILM VWorks form. The remaining automation protocols are executed from the RNA_XT_HS2_ILM VWorks form.



Prepare .csv file for normalizing pooled cDNA 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 cDNA 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 A\XT_HS2_RNA_ILM_v.Ax.x.x\Aliquot Input File Templates** (where x.x.x is the version number).

The Aliquot_Water_Template.csv file may be copied and used as a template for creating the .csv file for each **06b Aliquot_Water** protocol run. If you are using the sample file as a template for runs with fewer than 12 columns, be sure to delete rows for any unused columns on the plate.

3 Load the .csv file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\XT_HS2_RNA_ILM_v.Ax.x.x\Pooling and Normalization Templates**.

Prepare the NGS Bravo and reagent reservoir for protocol 06b Aliquot_Water

You can reuse the reservoir that was used in the **Cleanup_Pre-CapPCR** protocol (04a or 04b) or use a fresh reservoir.

At the end of the automation protocol, you may retain the reservoir for later use in the **06c PoolingConcentration_XT_HS2_ ILM** protocol if you are running that protocol today.

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

2 Prepare an Agilent deep well reservoir containing 30 mL of nuclease-free water.

Load the NGS Bravo

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

Table 50 Initial Bravo deck configuration for protocol 06b Aliquot_Water

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

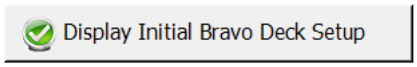
* The **06b Aliquot_Water** protocol does not use the Current Tip State indicator. The tip box at position 8 must be new and full of unused tips.

Run VWorks protocol 06b Aliquot_Water

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

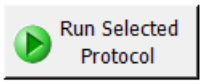
2 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.

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



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

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



6 When prompted, browse to the .csv file created for the source plate of the current run in **step 3** on **page 100**, and then click **OK** to start the run.

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

7 Remove the sample plate from the Bravo deck.

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

Prepare the NGS Bravo and reagent reservoirs for protocol 06c PoolingConcentration_XT_HS2_ ILM

You can reuse the reservoir that was used in the **06b Aliquot_Water** protocol or use fresh reservoirs.

At the end of the automation protocol, you may retain the reservoirs for later use in the **09 SSELWash_XT_HS2_ILM** protocol if you are running that protocol today.

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

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

3 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 deep well reservoir containing 30 mL of nuclease-free water.

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

Load the NGS Bravo

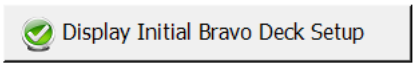
1 Load the Bravo deck according to **Table 51**.

Table 51 Initial Bravo deck configuration for protocol 06c PoolingConcentration_XT_HS2_ILM

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
3	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
5	Aliquotted AMPure XP beads in Agilent deep well plate from **page 42** (180 µL of beads/well)
6	Eppendorf twin.tec or Armadillo plate containing cDNA library pools from the **06b Aliquot_Water** protocol
8	Empty tip box
9	70% ethanol reservoir from **step 5**, above

Run VWorks protocol 06c PoolingConcentration_XT_HS2_ILM

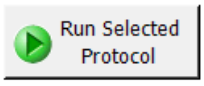
1. On the SureSelect setup form, under **Select protocol to execute**, select the **06c PoolingConcentration_XT_HS2_ILM** protocol.
2. Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.
3. Select the number of columns of samples to be processed.
4. Click **Display Initial Bravo Deck Setup**.



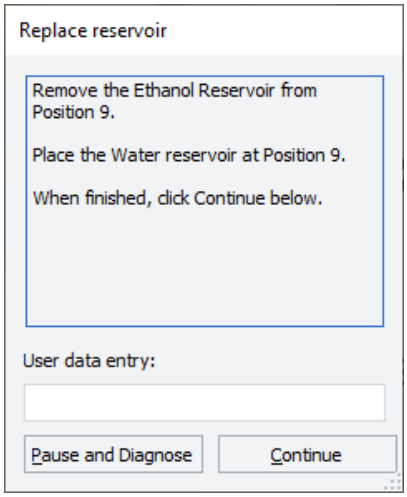
5. Verify that the Bravo deck has been set up as displayed on the right side of the form.
6. Upload the .csv file created in **step 1** through **step 3** on **page 99**.
   a. Click the "..." button below **Select Aliquot Input File** to open a directory browser window.

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

      The directory browser window closes, returning you to the SureSelect setup form. The selected file location is listed in the field below **Select Aliquot Input File**.
7. Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.
8. When verification is complete, click **Run Selected Protocol**.



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



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

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

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

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

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

Table 52 Reagents for Hybridization

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect XT HS2 Blocker Mix (blue cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	**page 105**
SureSelect RNase Block (purple cap)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	**page 106**
SureSelect Fast Hybridization Buffer (bottle)	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw and keep at Room Temperature	**page 106**
Probe	–80°C	Thaw on ice	**page 106**

Pre-program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with the appropriate program for the Probe.

- For the SureSelect XT HS Human All Exon V8 Probes and SureSelect XT HS PreCap Human All Exon V8 Probes – including V8 and V8+UTR – use the program in **Table 53**.
- For all other probes, use the program in **Table 54**.

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

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

Table 53 Hybridization program for *SureSelect XT HS and PreCap XT HS Human All Exon V8 Probes**

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

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

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

‡ Samples are held at 65°C until they are processed in the Capture automation protocol that begins on **page 113**.

Table 54 Hybridization program for *all other probes**

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

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

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

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

** Samples are held at 65°C until they are processed in the Capture automation protocol that begins on **page 113**.

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

NOTE

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

- In the final segment of the thermal cycler program (**Table 53** or **Table 54**), replace the 65°C Hold step with a 21°C Hold step.

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

Prepare the NGS Bravo for protocol 07 Hyb_XT_HS2_ILM

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
3. Pre-set the temperature of Bravo deck positions 4 and 6 to 23°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
4. Place a red PCR plate insert at Bravo deck position 4.

Prepare the Block master mix

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

   Table 55 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 55 Preparation of Block master mix

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

Prepare one or more Probe Hybridization master mixes

1 Prepare the appropriate volume of Probe Hybridization master mix for each of the Probes that will be used for hybridization as indicated in **Table 56** to **Table 59**. Mix by vortexing at medium speed for 15–20 seconds, then spin down briefly. Keep the master mix(es) on ice.

NOTE

Each row of the prepped cDNA 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 56** or **Table 57**) on **page 106**. The tables list the required reagent volumes for 1 to 12 columns of samples, across all 8 rows of wells.
- For runs that use different Probes for individual rows, prepare each master mix as described in Step b (**Table 58** or **Table 59**) on **page 107**.The tables list the required reagent volumes for 1 to 12 columns of samples, across a single row of wells.

---

a For runs that use a single Probe for all rows, prepare a master mix as described in **Table 56** or **Table 57**, according to the probe design size.

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

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

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

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

b For runs that use different Probes in individual rows, prepare a master mix for each Probe as listed in **Table 58** or **Table 59**, according to the probe design size. The volumes listed in **Table 58** and **Table 59** 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 58 Preparation of Probe Hybridization master mix for Probes <3 Mb, single row of wells

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

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

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

Prepare the Hybridization master mix source plate

1 Prepare the hybridization master mix source plate at room temperature, containing the master mixes prepared above (**Prepare the Block master mix** and **Prepare one or more Probe Hybridization master mixes**). Add the volumes indicated in **Table 60** of each master mix to each well of the indicated column of the plate. When using multiple Probes in a run, add the Probe Hybridization master mix for each probe to the appropriate row(s) of the processing plate. The final configuration of the master mix source plate is shown in **Figure 14**.

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

Table 60 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 60 Preparation of the master mix source plate for protocol 07 Hyb_XT_HS2_ILM

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

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

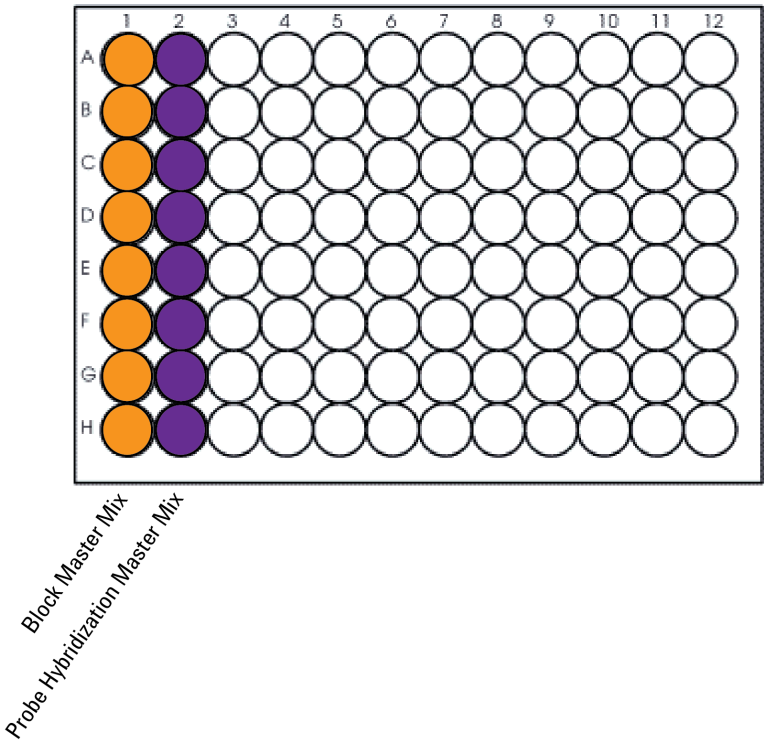


Figure 14 Configuration of the master mix source plate for protocol **07 Hyb_XT_HS2_ILM**. Column 2 can contain different Probe Hybridization master mixes in each row.

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

Load the Bravo deck

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

Table 61 Initial Bravo deck configuration for protocol 07 Hyb_XT_HS2_ILM

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

Run VWorks protocol 07 Hyb_XT_HS2_ILM

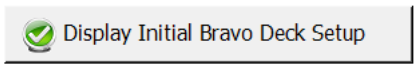
1 On the SureSelect setup form, under **Select protocol to execute**, select the **07 Hyb_XT_HS2_ILM** protocol.

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

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

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

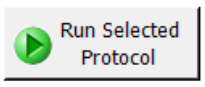
5 Click **Display Initial Bravo Deck Setup**.



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

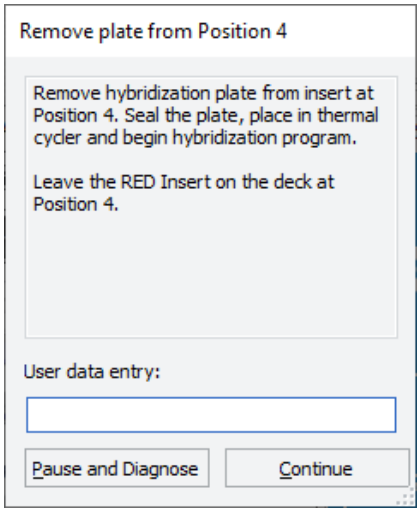
7 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



The NGS Bravo combines the prepped cDNA 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.

9 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**.



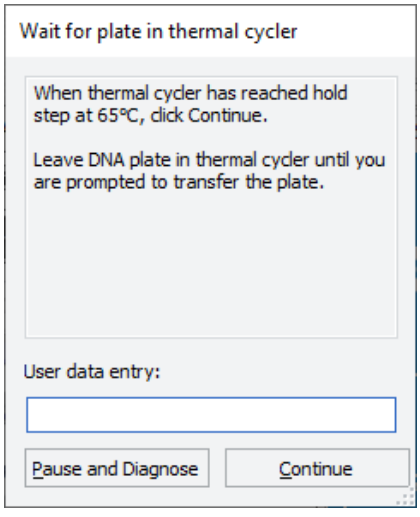
10 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

11 Transfer the sealed plate to a thermal cycler. Initiate the pre-programmed thermal cycler program (**Table 53** on page 104 or **Table 54** on page 104).

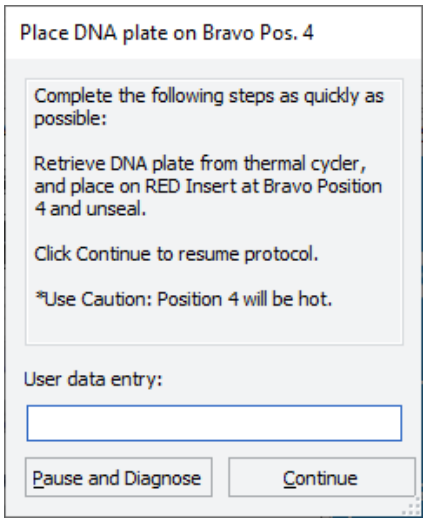
While the sample plate incubates on the thermal cycler, the NGS Bravo aliquots the Probe Hybridization master mix to the Eppendorf twin.tec or Armadillo plate.

CAUTION You must complete **step 12** to **step 16** quickly, and immediately after being prompted by the VWorks software. It is important that sample temperature remains approximately 65°C during transfers between the NGS Bravo and thermal cycler.

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



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

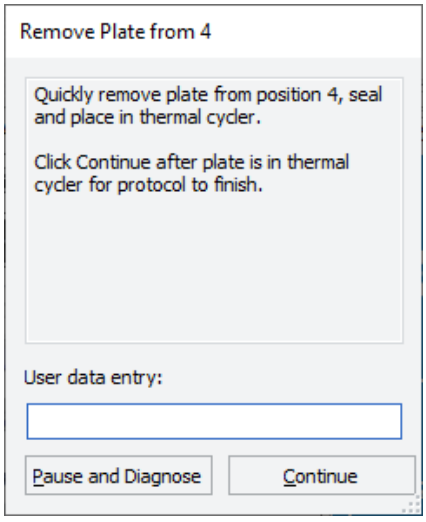


WARNING **Bravo deck position 4 will be hot.**

Use caution when handling components that contact heated deck positions.

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

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



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

16 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 53** on page 104 or **Table 54** on page 104). During this step, the prepared cDNA samples or cDNA 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.

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

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

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

Step 3. Capture the hybridized cDNA

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

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

If performing same-day hybridization and capture, setup tasks for the Capture protocol (**Prepare the NGS Bravo for protocol 08 SSELCapture_XT_HS2_ILM**, **Prepare the Dynabeads streptavidin beads source plate**, and **Prepare wash buffer source plates**) should be completed during the thermal cycler incubation for hybridization (approximately 1.5 to 2.5-hour duration) started on **page 112**. If performing next-day capture after an overnight hold at 21°C, begin these tasks on day 2.

This step uses the components listed in **Table 62**.

Table 62 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 114**
SureSelect Wash Buffer 1	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	**page 114**
SureSelect Wash Buffer 2	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT	**page 114**
Dynabeads MyOne Streptavidin T1	Follow storage recommendations provided by supplier (see **Table 1** on page 13)	**page 114**

Prepare the NGS Bravo for protocol 08 SSELCapture_XT_HS2_ILM

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

Prepare the Dynabeads streptavidin beads source plate

1. Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. The beads settle during storage.
2. Wash the magnetic beads.
   a. In a conical vial, combine the components listed in **Table 63**. The volumes below include the required overage.

Table 63 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 63 Magnetic bead washing mixture

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

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

c Put the vial into a magnetic separator device.

d Remove and discard the supernatant.

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

3 Resuspend the beads in SureSelect Binding buffer, according to **Table 64** below.

Table 64 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 64 Resuspension volumes for washed bead suspension

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

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

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

Prepare wash buffer source plates

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

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

Load the NGS Bravo

1 Load the Bravo deck according to **Table 65** (position 5 should already be loaded).

Table 65 Initial Bravo deck configuration for protocol 08 SSELCapture_XT_HS2_ILM

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
3	*Wash #1* source plate (Eppendorf twin.tec or Armadillo plate) from **step 1**, **page 114**
4	Empty red insert
5	Dynabeads streptavidin bead Deep Well source plate
6	*Wash #2* source plate (Agilent Deep Well plate) from **step 2**, **page 114**, seated on silver Deep Well insert
8	Empty tip box

Set up VWorks protocol 08 SSELCapture_XT_HS2_ILM

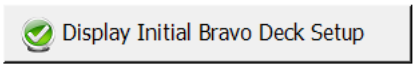
1 On the SureSelect setup form, under **Select protocol to execute**, select the **08 SSELCapture_XT_HS2_ILM** protocol.

2 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.

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

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

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



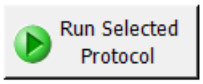
6 Verify that the Bravo deck has been set up as displayed on the right side of the form.

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

Run VWorks protocol 08 SSELCapture_XT_HS2_ILM

Start the **08 SSELCapture_XT_HS2_ILM** protocol upon completion of the hybridization incubation. The hybridization incubation is complete when the thermal cycler program reaches the 65°C Hold step. The 65°C Hold step is segment 6 if using **Table 53** on page 104, and it is segment 5 if using **Table 54** on page 104.

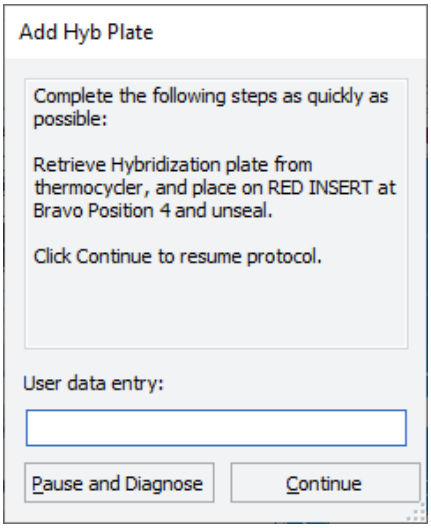
1 After verifying that the hybridization step is complete and that all NGS Bravo setup steps for capture are complete, click **Run Selected Protocol**. Leave the hybridization plate in the thermal cycler until you are prompted to transfer the plate to the NGS Bravo.



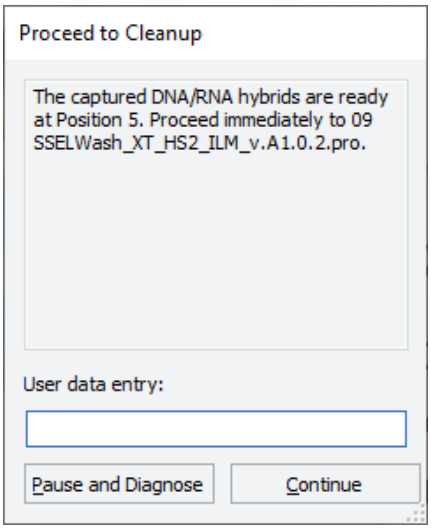
The total duration of the **08 SSELCapture_XT_HS2_ILM** protocol is approximately 35 minutes. An operator must be present to transfer the hybridization plate from the thermal cycler when prompted by VWorks as shown in **step 2** below (<5 minutes after starting the protocol).

CAUTION It is important to complete **step 2** quickly and carefully. Unseal the plate without tilting or jerking the plate to avoid sample splashing. Make sure that the NGS Bravo is completely prepared, with all components in place, before you transfer the sample plate to the Bravo deck.

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



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


Proceed to Cleanup
The captured DNA/RNA hybrids are ready at Position 5. Proceed immediately to 09 SSELWash_XT_HS2_ILM_v.A1.0.2.pro.
User data entry:
Pause and Diagnose
Continue

Step 4. Wash the captured cDNA

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

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

Prepare the NGS Bravo and reagent reservoir for protocol 09 SSELWash_XT_HS2_ILM

At the end of the automation protocol, you may retain the reservoir for later use in the **10 Post-CapPCR_XT_HS2_ILM** protocol if you are running that protocol today.

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

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

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

2 Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in **Table 66**. See **Setting the Temperature of Bravo Deck Heat Blocks** for more information on how to do this step.

Table 66 Bravo Deck Temperature Presets for protocol 09 SSELWash_XT_HS2_ILM

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

3 Prepare an Agilent deep well reservoir containing 30 mL of nuclease-free water.

Load the NGS Bravo

1 Load the Bravo deck according to **Table 67** (positions 3, 4, 5, and 6 should already be loaded).

Table 67 Initial Bravo deck configuration for protocol 09 SSELWash_XT_HS2_ILM

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

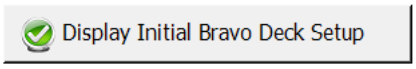
Run VWorks protocol 09 SSELWash_XT_HS2_ILM

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

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

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

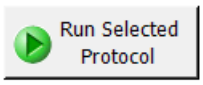
4 Click **Display Initial Bravo Deck Setup**.



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

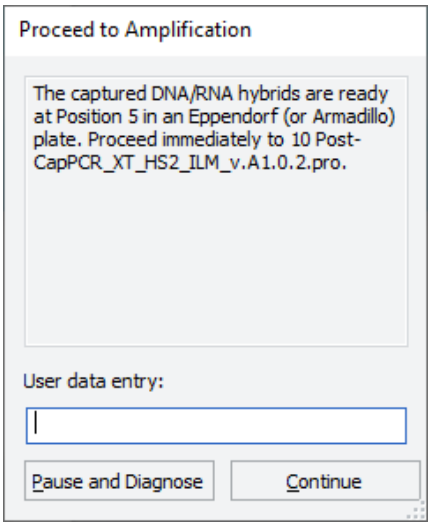
6 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



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

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



Proceed immediately to the **10 Post-CapPCR_XT_HS2_ILM** protocol, starting on **page 122**.

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

7 Post-Capture Sample Processing for Multiplexed Sequencing

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

Step 1. Amplify the captured libraries

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

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

The design size of your Probe determines the amplification cycle number. Plan your experiments for amplification of samples prepared using probes of similar design sizes on the same plate. See **Table 70** on page 123 for cycle number recommendations.

This step uses the components listed in **Table 68**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 68 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 124**
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 124**
SureSelect Post-Capture Primer Mix (clear cap)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Vortexing	**page 124**

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

Prepare the NGS Bravo and reagent reservoir for protocol 10 Post-CapPCR_XT_HS2_ILM

You can reuse the reservoir that was used in the **09 SSELWash_XT_HS2** protocol or use a fresh reservoir.

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
3. Prepare an Agilent deep well reservoir containing 30 mL of nuclease-free water.
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 69**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

Table 69 Post-capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	12 to 16 See **Table 70** 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 70 Post-capture PCR cycle number recommendations

Probe Size/Description	Cycles
Probes <0.2 Mb	16 cycles
Probes 0.2–3 Mb	14 cycles
Probes 3–5 Mb	13cycles
Probes >5 Mb (including Human All Exon Probes)	12 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 71**. Mix well using a vortex mixer and keep on ice.

Table 71 lists the required reagent volumes for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 71 Preparation of Post-Capture PCR master mix

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

2 Using the same master mix source plate (Eppendorf twin.tec or Armadillo plate) that was used for the **07 Hyb_XT_HS2_ILM** protocol run, prepare the master mix source plate by adding the volume of PCR master mix indicated in **Table 72** to all wells of column 3 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 15**.

Table 72 lists the master mix volume for 1 to 12 columns of samples. Make sure to use the volumes appropriate for your run.

Table 72 Preparation of the master mix source plate for protocol 10 Post-CapPCR_XT_HS2_ILM

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

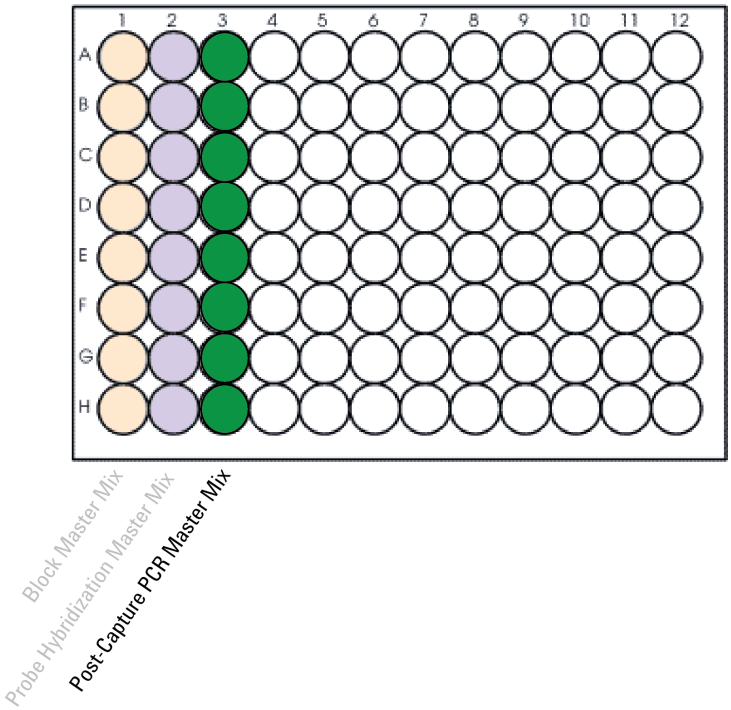


Figure 15 Configuration of the master mix source plate for protocol **10 Post-CapPCR_XT_HS2_ILM**. The master mixes dispensed during previous protocols are shown in light shading.

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 Bravo

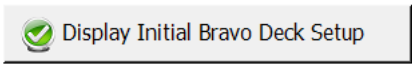
1 Load the Bravo deck according to **Table 73**.

Table 73 Initial Bravo deck configuration for protocol 10 Post-CapPCR_XT_HS2_ILM

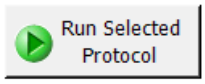
Location	Content
1	Nuclease-free water reservoir from **step 3**, **page 122**
2	New tip box
5	Captured DNA bead suspensions in Eppendorf twin.tec or Armadillo plate (unsealed)
6	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
8	Empty tip box
9	Master mix source plate containing PCR Master Mix in Column 3 (unsealed)

Run VWorks protocol 10 Post-CapPCR_XT_HS2_ILM

1. On the SureSelect setup form, under **Select protocol to execute**, select the **10 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. Verify that the **Processing Plate** selection is set to the correct plate type. See **page 23** for more information on using this segment of the form.
4. Select the number of columns of samples to be processed.
5. Click **Display Initial Bravo Deck Setup**.

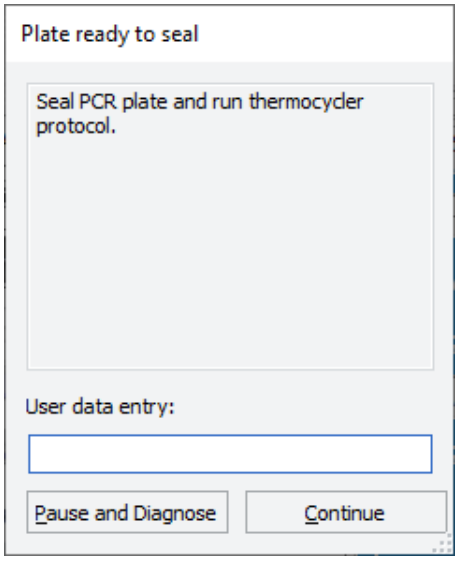


6. Verify that the NGS Bravo has been set up as displayed on the right side of the form.
7. Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.
8. When verification is complete, click **Run Selected Protocol**.



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

9. 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.



10. Place the plate in the thermal cycler. Resume the thermal cycler program in **Table 69** on page 123.
11. When the PCR amplification program is complete, spin the plate briefly then keep on ice.

Retain the Eppendorf twin.tec or Armadillo source plate containing the Post-Capture PCR master mix located at position 9 of the Bravo deck for later use in the **12 TS_HighSensitivity_D1000** protocol (see **"Option 1: Analysis using the Agilent 4200 TapeStation and High Sensitivity D1000 ScreenTape"** on page 131).

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

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

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

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

Prepare the NGS Bravo and reagent reservoir for protocol 11 Cleanup_Post-CapPCR_XT_HS2_ILM

You can reuse the reservoir that was used in the **10 Post-CapPCR_XT_HS2_ILM** protocol or use fresh reservoirs.

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in **Table 74**. See **Setting the Temperature of Bravo Deck Heat Blocks** for more information on how to do this step.

Table 74 Deck Temperature Presets for protocol 11 Cleanup_Post-CapPCR_XT_HS2_ILM

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

3. Prepare an Agilent deep well reservoir containing 30 mL of nuclease-free water.
4. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.

Load the NGS Bravo

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

Table 75 Initial Bravo deck configuration for protocol 11 Cleanup_Post-CapPCR_XT_HS2_ILM

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
2	New tip box
3	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
5	Aliquotted AMPure XP beads in Agilent Deep Well plate from **page 44** (50 µL of beads/well)
6	Amplified DNA libraries or library pools in unsealed PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
8	Empty tip box
9	70% ethanol reservoir from **step 4**

Run VWorks protocol 11 Cleanup_Post-CapPCR_XT_HS2_ILM

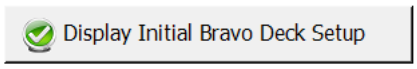
1 On the SureSelect setup form, under **Select protocol to execute**, select the **11 Cleanup_Post-CapPCR_XT_HS2_ILM** protocol.

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

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

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

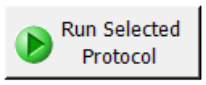
5 Click **Display Initial Bravo Deck Setup**.



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

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

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



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

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

Step 3. Assess sequencing library DNA quantity and quality

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

- Option 1: Prepare the analytical assay plate using automation (protocol **12 TS_HighSensitivity_D1000**) and perform analysis on Agilent 4200 TapeStation. See **"Option 1: Analysis using the Agilent 4200 TapeStation and High Sensitivity D1000 ScreenTape"** on page 131.
- 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 136.

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

This section describes use of automation protocol **12 TS_HighSensitivity_D1000** to prepare samples for analysis. The automation protocol prepares the assay sample plate by combining 3 µL of each DNA sample with 3 µL of High Sensitivity D1000 Sample Buffer. Afterward, you transfer the sample plate to the 4200 TapeStation instrument for analysis. For more information to do this step, see the Agilent High Sensitivity D1000 Assay Quick Guide for 4200 TapeStation System.

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

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

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Turn off the ThermoCube device (see **page 22**) to restore position 9 of the Bravo deck to room temperature.
3. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Using the same master mix source plate that was used for the **10 Post-CapPCR_XT_HS2_ILM** protocol run, prepare the Sample Buffer source plate at room temperature. Add the volume of High Sensitivity D1000 Sample Buffer indicated in **Table 76** to each well of column 4 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 16**.

   Table 76 lists the required master mix volumes for 1 to 12 columns of samples. Make sure to use the volume appropriate for your run.

Table 76 Preparation of the Sample Buffer Source Plate for protocol 12 TS_HighSensitivity_D1000

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

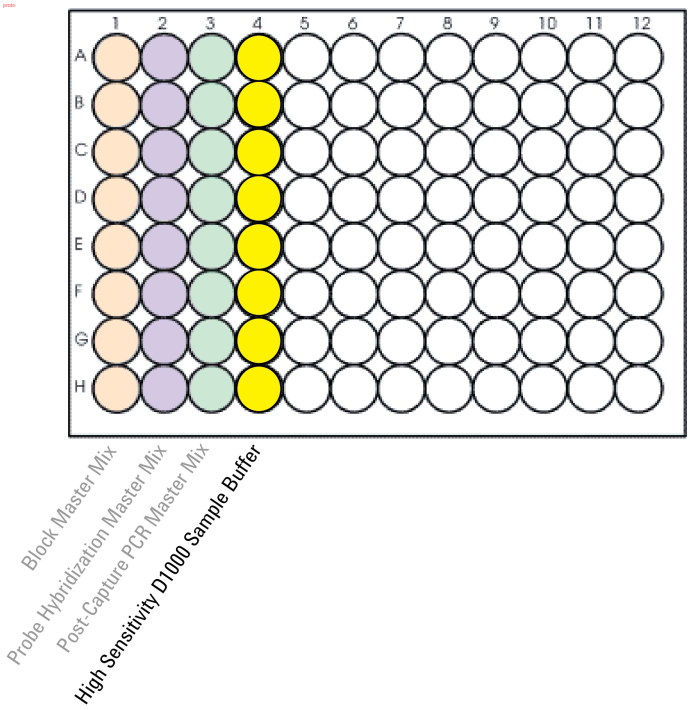


Figure 16 Configuration of the source plate for protocol **12 TS_HighSensitivity_D1000**. Columns 1–3 were used to dispense master mixes during previous protocols.

Load the NGS Bravo

5 Load the Bravo deck according to **Table 77**.

Table 77 Initial Bravo deck configuration for protocol 12 TS_HighSensitivity_D1000

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

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

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

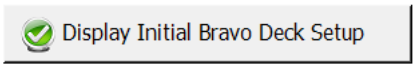
Run VWorks protocol 12 TS_HighSensitivity_D1000

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

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

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

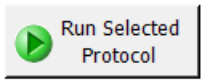
9 Click **Display Initial Bravo Deck Setup**.



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

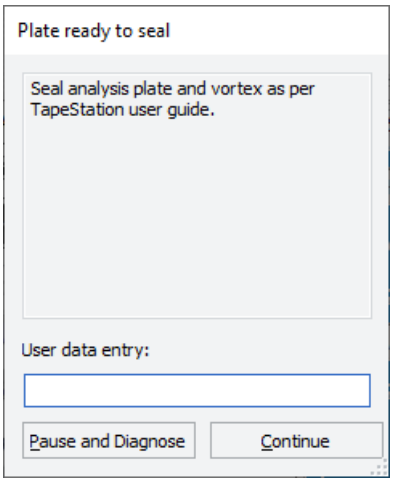
11 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 24** for more information on using this segment of the form during the run.

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



Running the **12 TS_HighSensitivity_D1000** protocol takes approximately 10 minutes. Once removal of analytical samples is complete, remove the primary DNA library sample plate from position 4, seal the plate, and keep on ice until the samples are pooled for sequencing on **page 137**.

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



CAUTION

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

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

Run the High Sensitivity D1000 Assay and analyze the data

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

15 Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 78** for guidelines). Sample electropherograms are shown in **Figure 17** (library prepared from high-quality RNA) and **Figure 18** (library prepared from typical FFPE RNA).

Table 78 Post-capture library qualification guidelines

Input RNA type	Expected library DNA fragment size peak position	NGS read lengths supported
High-quality RNA or FFPE RNA	200 to 700 bp	2 × 100 reads or 2 × 150 reads

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

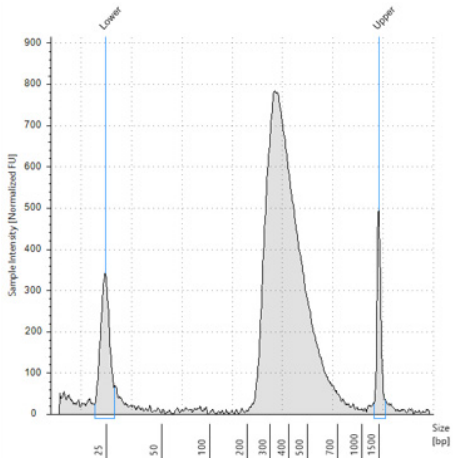


Figure 17 Post-capture library prepared from an intact RNA sample analyzed using a High Sensitivity D1000 ScreenTape assay

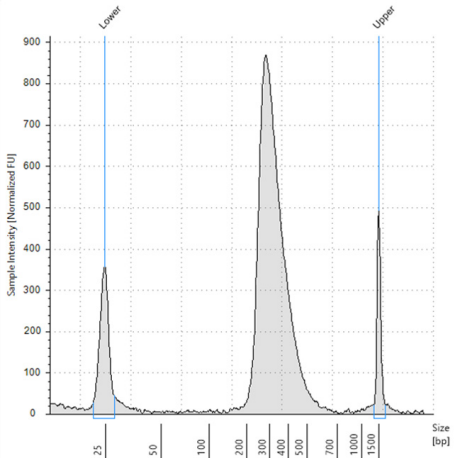


Figure 18 Post-capture library prepared from a typical FFPE RNA sample analyzed using a High Sensitivity D1000 ScreenTape assay

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 17** and **Figure 18**). Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 78** for guidelines). **Table 79** includes links to assay instructions.

Table 79 Post-capture library analysis options

Analysis platform	Assay used at this step	Link to assay instructions	Amount of library sample to analyze
Agilent 4200 or 4150 TapeStation system	High Sensitivity D1000 ScreenTape	Agilent High Sensitivity D1000 Assay Quick Guide	2 µL
Agilent 2100 Bioanalyzer system	High Sensitivity DNA Kit	Agilent High Sensitivity DNA Kit Guide	1 µL
Agilent 5200, 5300, or 5400 Fragment Analyzer system	HS NGS Fragment Kit (1-6000 bp)	Agilent HS NGS Fragment Kit (1-6000 bp) Guide	2 µL

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

Step 4. Pool samples for multiplexed sequencing (optional)

NOTE

Pre-Capture Pooling Workflow If you are using the pre-capture pooling workflow, then your samples were already pooled prior to hybridization with the Probe. The final captured DNA samples contain pools of either 8 or 16 indexed libraries, based on the Probe used and resulting pre-capture pooling strategy. When appropriate for your sequencing platform, the 8-plex or 16-plex samples may be further multiplexed by post-capture pooling. Determine whether to do post-capture pooling by calculating the number of indexes that can be combined per lane, according to the output specifications of the platform used, together with the amount of sequencing data required for your research design. If doing post-capture pooling, use the guidelines provided below. If samples will not be further combined in post-capture pools, proceed to **"Step 5. Prepare sequencing samples"** on page 141.

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

Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool using one of the two methods described below. **Method 2** can use the **13 Aliquot_Captures** automation protocol to pool samples.

Method 1 Dilute each indexed library to be pooled to the same final concentration (typically 4 nM to 15 nM, or the concentration of the most dilute sample) using Low TE. This dilution step is performed by manually pipetting the Low TE directly into the wells of the source plate. Then, combine equal volumes of all libraries to create the final pool in the destination plate.

Method 2 Starting with indexed libraries at different concentrations, add the appropriate volume of each library to the destination well (either manually or using the **13 Aliquot_Captures** automation protocol) to achieve equimolar concentration in the pool. Then, adjust the pool to the desired final volume by adding the appropriate volume of Low TE to each well. This volume adjustment is performed by manually pipetting the Low TE directly into the wells of the destination plate. The formula below is provided for determination of the amount of each indexed sample to add to the pool.

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

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

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

is the number of indexes, and

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

Table 80 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 80 Example of volume calculation for total volume of 20 µL at 10 nM concentration

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

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

The instructions below are for **Method 2**. Alternatively, you can perform this method using an entirely manual approach rather than with the **12 Aliquot_Captures** automation protocol.

1. Create a .csv (comma separated value) file with the headers shown in **Figure 19**. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in .csv format. The file must include rows for all 96 wells of the plate.
2. Enter the information requested in the header for each DNA sample. **Figure 19** shows an example spreadsheet.
   - In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
   - In the SourceWell field, enter each well position on the source plate containing an amplified and captured indexed library that needs to be added to a pool. Use the Eppendorf twin.tec plate containing the purified indexed libraries as the source plate.
   - In the DestinationWell field, enter the well position on the destination plate for the pool.
   - In the Volume field, enter the volume (in µL) of each indexed library to be transferred from the source well to the destination well. The volume for each library is calculated from its concentration.
   - For all empty wells on the source plate, delete the corresponding rows in the .csv file.

	A	B	C	D
1	SourceBC	SourceWell	DestinationWell	Volume
2	abc	A1	A1	4.711292
3	abc	B1	A1	6.37105
4	abc	C1	A1	7.000448
5	abc	D1	A1	3.81144
6	abc	E1	A1	9.539072
7	abc	F1	A1	7.802747
8	abc	G1	A1	8.835171
9	abc	H1	A1	6.313131
10	abc	A2	A1	5.976286
11	abc	B2	A1	6.601183
12	abc	C2	A1	7.14449
13	abc	D2	A1	5.66431

Figure 19 Sample spreadsheets for method 1 and method 2

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option A\XT_HS2_RNA_ILM_v.Ax.x.x\Aliquot Input File Templates\Aliquot_Captures_Template.csv** (where x.x.x is the version number).

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

3 Load the .csv file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option A\XT_HS2_RNA_ILM_v.Ax.x.x\Aliquot Input File Templates**.

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

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

6 Load the Bravo deck according to **Table 81**.

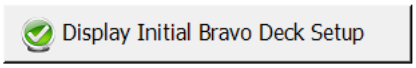
Table 81 Initial Bravo deck configuration for protocol 13 Aliquot_Captures

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

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

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

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



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

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

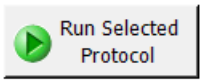
12 Upload the .csv file created in **step 1** through **step 3**.

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

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

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

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



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

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

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

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

Step 5. Prepare sequencing samples

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

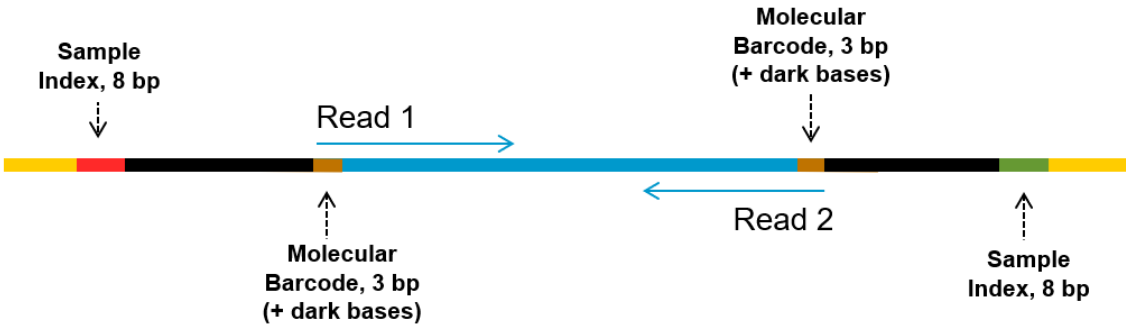


Figure 20 Content of SureSelect XT HS2 sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the dual sample indexes (red and green), molecular barcodes (brown) and the library PCR primers (yellow).

Libraries can be sequenced on the Illumina HiSeq, MiSeq, NextSeq or NovaSeq platform using the run type and chemistry combinations shown in **Table 82**.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See **Table 82** for kit configurations compatible with the recommended read length.

The optimal seeding concentration for SureSelect XT HS2 target-enriched libraries varies according to sequencing platform, run type, and Illumina kit version. See **Table 82** for guidelines. Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in **Table 82**.

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

Table 82 Illumina Kit Configuration Selection Guidelines

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

Step 6. Do the sequencing run and analyze the data

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

- Each of the sample-level indexes requires an 8-bp index read. For complete index sequence information, see **Table 112** on page 151 through **Table 118** on page 157.
- For the HiSeq, NextSeq, and NovaSeq platforms, set up the run using the instrument's user interface, following the guidelines on **page 143**.
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed on **page 144** to **page 146** to generate a custom sample sheet.
- Do not use the adaptor trimming options in Illumina Experiment Manager (IEM). Make sure any IEM adaptor trimming option checkboxes are cleared (deselected) when setting up the sequencing run. Adaptors are trimmed in later processing steps as described below to ensure proper processing of the degenerate MBCs in the adaptor sequences.
- Demultiplex using Illumina's bcl2fastq, BCL Convert or DRAGEN software to generate paired end reads based on the dual indexes and remove sequences with incorrectly paired P5 and P7 indexes. Do not use the MBC/UMI trimming options in Illumina's demultiplexing software if using Agilent's Genomics NextGen Toolkit (AGeNT) or SureCall software to process your FASTQ files.
- Before aligning reads to the reference genome, Illumina adaptor sequences should be trimmed from the reads using Agilent's AGeNT Trimmer module, which properly accounts for the degenerate MBCs in the adaptor sequence. See **page 147** for more information.
- Library fragments include a degenerate molecular barcode (MBC) in each strand (see **Figure 20** on page 141). Note that unlike DNA, where both strands are present and the MBCs in the strands can be matched to form a duplex consensus read, analysis of single- stranded RNA is limited to consensus generation using the MBC from one strand.
- The MBC sequence and dark bases are located at the 5' end of both Read 1 and Read 2. Agilent recommends using AGeNT for barcode extraction and trimming (see **page 147** for more information). If your sequence analysis pipeline excludes MBCs and is incompatible with AGeNT, you can trim or mask the first five bases from each read before alignment as described in the *Note* on **page 147**.

HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface, using the settings shown in **Table 83**. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface and enter the Cycles settings in **Table 83**.

For the NextSeq or NovaSeq platform, open the *Run Setup* screen of the instrument control software interface and enter the **Read Length** settings in **Table 83**. In the **Custom Primers** section, clear (do **not** select) the check boxes for all primers (*Read 1, Read 2, Index 1* and *Index 2*).

Table 83 Run settings

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

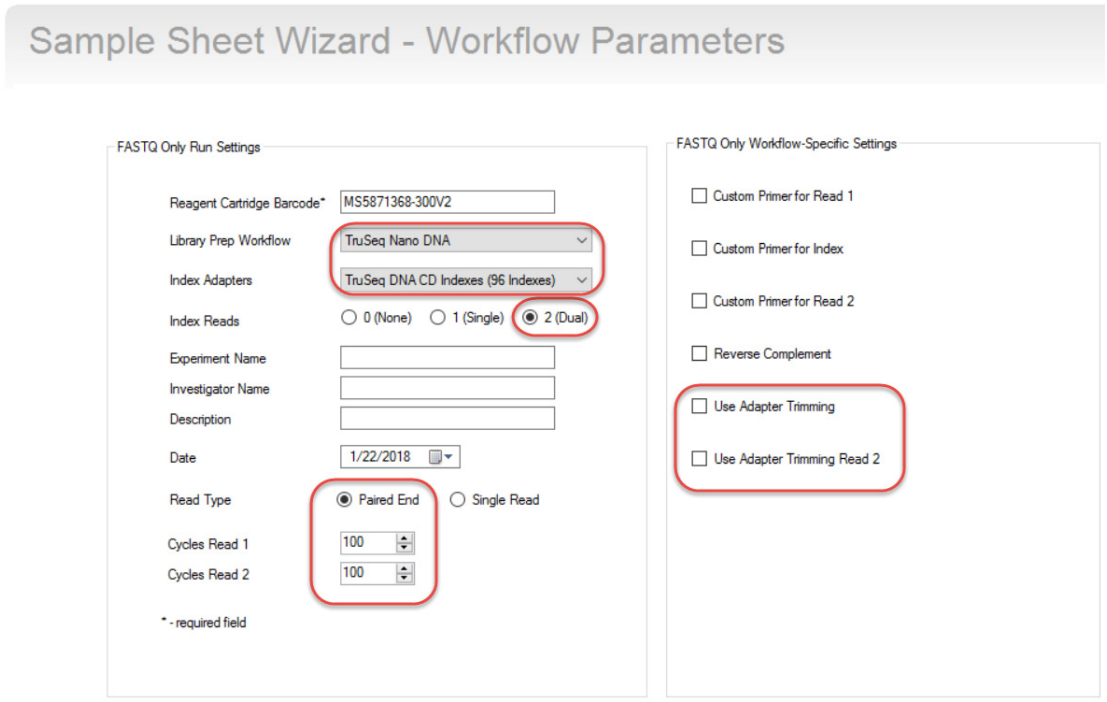
MiSeq platform sequencing run setup guidelines

Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below. Once a Sample Sheet has been generated, index sequences need to be manually changed to the SureSelect XT HS2 indexes used for each sample. See **Table 91** on page 153 through **Table 98** on page 160 for nucleotide sequences of the SureSelect XT HS2 index pairs.

Set up a custom Sample Sheet:

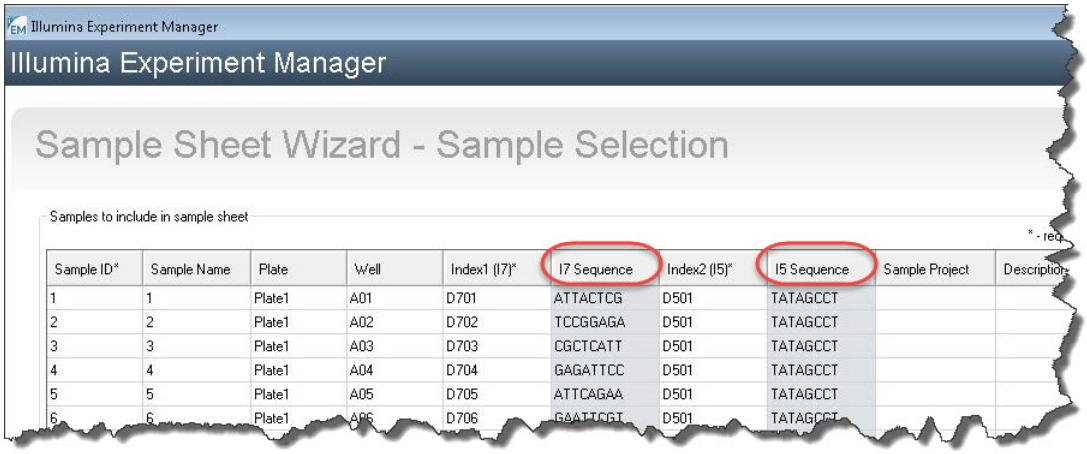
1. In the IEM software, create a Sample Sheet for the MiSeq platform using the following Workflow selections.
   - Under **Category**, select *Other*.
   - Under **Application**, select *FASTQ Only*.
2. On the **Workflow Parameters** screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA.** In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. Clear both adaptor-trimming checkboxes under *FASTQ Only Workflow-Specific Settings*, since adaptor trimming must be performed using Agilent's AGeNT software (see **page 147**).

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



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

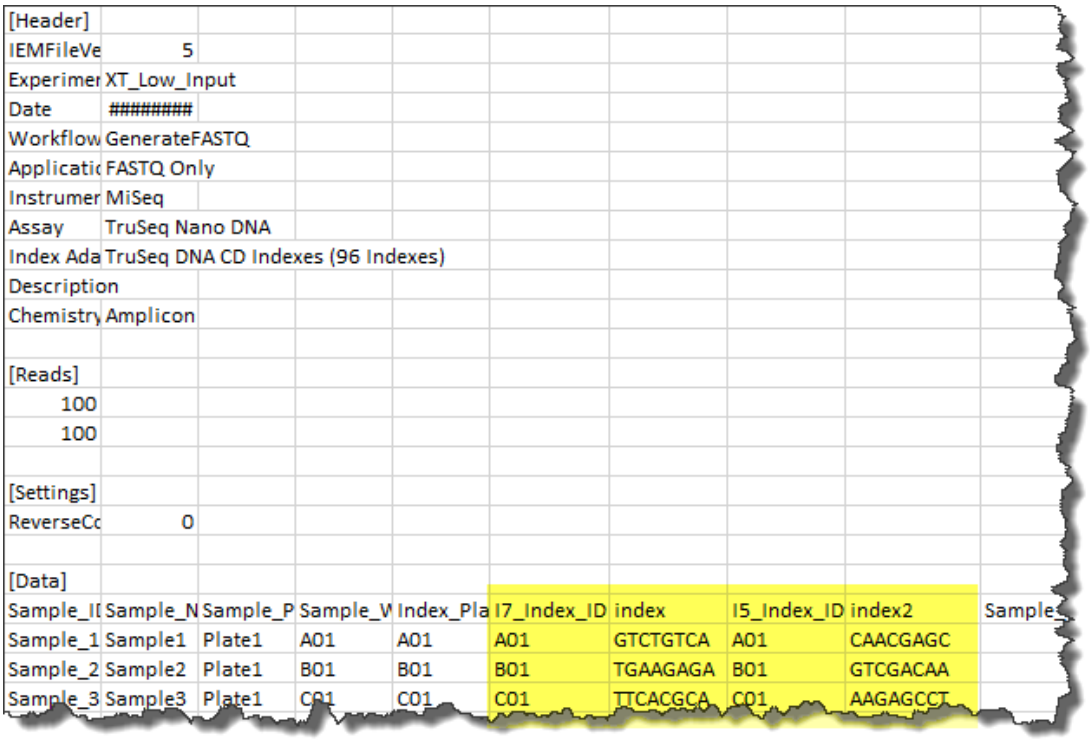
Likewise, in the **I5 Sequence** column, assign any of the Illumina i5 indexes, to be corrected to the i5 sequence from the SureSelect XT HS2 index pair at a later stage.



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

Editing the Sample Sheet to include SureSelect XT HS2 dual indexes

- Open the Sample Sheet file in a text editor and edit the i7 and i5 index information for each sample in columns 5–8 (highlighted below). See see **Table 91** on page 153 through **Table 98** on page 160 for nucleotide sequences of the SureSelect XT HS2 indexes.
- In column 5 under **I7_Index_ID**, enter the SureSelect XT HS2 index pair number assigned to the sample. In column 6 under **index**, enter the corresponding P7 index sequence.
- In column 7 under **I5_Index_ID**, enter the SureSelect XT HS2 index pair number assigned to the sample. In column 8 under **index2**, enter the corresponding P5 index sequence.
- If the run includes more than 96 samples, the sample sheet may be edited to include additional sample rows containing the assigned SureSelect XT HS2 index pair sequences in column 6 (P7 index) and column 8 (P5 index).



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

Figure 21 Sample sheet for SureSelect XT HS2 library sequencing

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

Sequence analysis resources

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

Use Illumina's bcl2fastq, BCL Convert or DRAGEN software to generate paired end reads by demultiplexing sequences based on the dual indexes and to remove sequences with incorrectly paired P5 and P7 indexes.

The demultiplexed FASTQ data needs to be pre-processed to remove sequencing adaptors and extract the molecular barcode (MBC) sequences using the Agilent Genomics NextGen Toolkit (AGeNT). AGeNT is a set of Java-based software modules that provide MBC pre- processing, adaptor trimming and duplicate read identification. This toolkit is designed to enable building, integrating, maintaining, and troubleshooting internal analysis pipelines for users with bioinformatics expertise. For additional information and to download this toolkit, visit the **AGeNT page at www.agilent.com** and review the **AGeNT Best Practices** document for processing steps suitable for XT HS2 RNA libraries.

NOTE

If your sequence analysis pipeline excludes MBCs, you can remove the first 5 bases from Read 1 and Read 2 by masking or trimming before proceeding to downstream analysis. If demultiplexing using bcl2fastq, MBCs may be masked by including the base mask **N5Y*,I8,I8,N5Y*** (where * is replaced with the actual read length, matching the read length value in the RunInfo.xml file). If demultiplexing using BCL Convert, MBCs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y*;I8;I8;N5Y*** (where * is replaced with read length after trimming, e.g., use N5Y145;I8;I8;N5Y145 for 2x150 NGS).

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

The trimmed reads should be aligned using a suitable RNA data alignment tool. Once alignment is complete, the AGeNT CReaK (Consensus Read Kit) tool can be used in the single-strand consensus mode to generate consensus reads and mark or remove duplicates. The resulting BAM files are ready for downstream analysis including gene expression and 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.

Strandedness guidelines

The SureSelect XT HS2 RNA sequencing library preparation method preserves RNA strandedness using dUTP second- strand marking. The sequence of read 1, which starts at the P5 end, matches the reverse complement of the poly-A RNA transcript strand. Read 2, which starts at the P7 end, matches the poly-A RNA transcript strand. When running analysis of this data to determine strandedness, it is important to include this information. For example, when using the Picard tools (**https://broadinstitute.github.io/picard**) to calculate RNA sequencing metrics, it is important to include the parameter *STRAND_SPECIFICITY=SECOND_READ_TRANSCRIPTION_STRAND* to correctly calculate the strand specificity metrics.

8 Reference

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

Kit Contents

The SureSelect XT HS2 RNA System protocol using the Agilent NGS Bravo uses the kits listed in **Table 84**. Detailed contents of each of the multi-part component kits listed in **Table 84** are shown in **Table 86** through **Table 89** on the following pages.

Table 84 Component Kits

Kit Name (p/n)	Component Kit Name	Component Kit p/n	Storage Condition
SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), 96 Reactions (G9993A through G9993D)	SureSelect cDNA Module (Pre PCR)	5500-0149	–20°C
	SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR)	5500-0151	–20°C
	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	5191-5688 (Index Pairs 1–96), 5191-5689 (Index Pairs 97–192), 5191-5690 (Index Pairs 193–288), OR 5191-5691 (Index Pairs 289–384)	–20°C
SureSelect XT HS2 RNA Target Enrichment Kit, 12 Hybs (G9994A)	SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	5191-6689	Room Temperature
	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	5191-6690	–20°C
SureSelect XT HS2 RNA Reagent Kit, 96 Reactions (G9991A through G9991D; or G9992A through G9992D with AMPure XP/ Streptavidin Beads)	SureSelect cDNA Module (Pre PCR)	5500-0149	–20°C
	SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR)	5500-0151	–20°C
	SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	5191-5688 through 5191-5691	–20°C
	SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR)	5190-9687	Room Temperature
	SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	5191-6688	–20°C
	SureSelect RNA AMPure® XP Beads (included with kits G9992A through G9992D)	5191-6671	+4°C
	SureSelect Streptavidin Beads (included with kits G9992A through G9992D)	5191-5742	+4°C

Table 85 SureSelect cDNA Module (Pre PCR) Content

Kit Component	96 Reaction Kit Format
2X Priming Buffer	tube with purple cap
First Strand Master Mix*	amber tube with amber cap
Second Strand Enzyme Mix	bottle
Second Strand Oligo Mix	tube with yellow cap

* The First Strand Master Mix contains actinomycin-D. Keep the reagent in the supplied amber vial to protect the contents from exposure to light.

Table 86 SureSelect XT HS2 RNA 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
XT HS2 RNA Adaptor Oligo Mix	tube with green cap
Herculase II Fusion DNA Polymerase	tube with red cap
5× Herculase II Reaction Buffer with dNTPs	tube with clear cap

Table 87 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 88 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 89 SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR) Content

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

SureSelect XT HS2 Index Primer Pair Information

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

The nucleotide sequence of the index portion of each primer is provided in **Table 91** through **Table 98**. P7 indexes are shown in forward orientation, applicable to any of the supported Illumina platforms. P5 indexes are shown in two orientations (forward and reverse complement) for use with different platforms and sequencing run setup and management tools, e.g., Local Run Manager and Instrument Run Setup. Illumina sequencing platforms and their P5 sequencing orientation are shown in **Table 90**. Correct representation of the P5 index orientation in sample sheets or during sequencing run setup is crucial to successful demultiplexing. Refer to Illumina support documentation and resources to determine the correct P5 index orientation for your application.

Table 90 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 91 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 92 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 93 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 94 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 95 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 96 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 97 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 98 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 99 Plate map for SureSelect XT HS2 Index Primer Pairs 1-96, provided in orange plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Table 100 Plate map for SureSelect XT HS2 Index Primer Pairs 97-192, provided in blue plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	1110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Table 101 Plate map for SureSelect XT HS2 Index Primer Pairs 193-288, provided in green plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	193	201	209	217	225	233	241	249	257	265	273	281
B	194	202	210	218	226	234	242	250	258	266	274	282
C	195	203	211	219	227	235	243	251	259	267	275	283
D	196	204	212	220	228	236	244	252	260	268	276	284
E	197	205	213	221	229	237	245	253	261	269	277	285
F	198	206	214	222	230	238	246	254	262	270	278	286
G	199	207	215	223	231	239	247	255	263	271	279	287
H	200	208	216	224	232	240	248	256	264	272	280	288

Table 102 Plate map for SureSelect XT HS2 Index Primer Pairs 289-384, provided in red plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	289	297	305	313	321	329	337	345	353	361	369	377
B	290	298	306	314	322	330	338	346	354	362	370	378
C	291	299	307	315	323	331	339	347	355	363	371	379
D	292	300	308	316	324	332	340	348	356	364	372	380
E	293	301	309	317	325	333	341	349	357	365	373	381
F	294	302	310	318	326	334	342	350	358	366	374	382
G	295	303	311	319	327	335	343	351	359	367	375	383
H	296	304	312	320	328	336	344	352	360	368	376	384

Quick Reference Tables for Master Mixes and Source Plates

This section contains copies of the tables for master mix formulations and source plate volumes used in the SureSelect XT HS2 RNA Protocol using NGS Bravo Option A protocol.

cDNA Synthesis

Table 103Fragmentation master mix source plate - used on page 51

Solution	Position on Source Plate	Volume (µL) of master mix added per Well of Agilent Deep Well Source Plate Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
2X Priming Buffer (tube with purple cap)	Column 1 (A1-H1)	19.8	31.8	43.8	55.8	67.8	79.8	91.7	103.8	115.9	128.0	140.1	152.2

Table 104 First Strand cDNA master mix source plate - used on page 55

Solution	Position on Source Plate	Volume (µL) of master mix added per Well of Agilent Deep Well Source Plate Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
First Strand Master Mix (amber tube with amber cap)	Column 2 (A2-H2)	16.8	27.0	37.2	47.4	57.6	67.8	78.0	88.3	98.5	108.8	119.1	129.4

Table 105Second Strand master mix - used on page 59

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Second Strand Enzyme Mix (bottle)	25 µL	310.5	550.8	791.2	1031.5	1367.5	1610.1	1852.6	2095.2	2337.7	2580.3	2822.8	3065.4
Second Strand Oligo Mix (tube with yellow cap)	5 µL	62.1	110.2	158.2	206.3	273.5	322.0	370.5	419.0	467.5	516.1	564.6	613.1
Total Volume	**30 µL**	**372.8**	**661.0**	**949.4**	**1237.8**	**1641.0**	**1932.1**	**2223.2**	**2514.2**	**2805.3**	**3096.3**	**3387.4**	**3678.5**

Table 106Second Strand master mix source plate - used on page 59

Solution	Position on Source Plate	Volume (µL) of master mix added per Well of Agilent Deep Well Source Plate Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Second Strand Master Mix	Column 3 (A3-H3)	44.4	78.7	113.0	147.4	195.4	230.0	264.7	299.3	334.0	368.6	403.3	437.9

Library Preparation

Table 107 End Repair/dA-Tailing master mix - used on page 67

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
End Repair-A Tailing Buffer (bottle)	16 µL	204	340	476	612	748	884	1042.7	1201.3	1360	1518.7	1677.3	1836
End Repair-A Tailing Enzyme Mix (tube with orange cap)	4 µL	51	85	119	153	187	221	260.7	300.3	340	379.7	419.3	459
Total Volume	**20 µL**	**255**	**425**	**595**	**765**	**935**	**1105**	**1303.4**	**1501.6**	**1700**	**1894.4**	**2096.6**	**2295**

Table 108 Ligation master mix - used on page 68

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Ligation Buffer (bottle)	23 µL	293.3	488.8	684.3	879.8	1075.3	1270.8	1515.1	1759.5	2003.9	2248.3	2492.6	2737
T4 DNA Ligase (tube with blue cap)	2 µL	25.5	42.5	59.5	76.5	93.5	110.5	127.5	153.0	174.3	195.5	216.8	238
Total Volume	**25 µL**	**318.8**	**531.3**	**743.8**	**956.3**	**1168.8**	**1381.3**	**1642.6**	**1912.5**	**2178.2**	**2443.8**	**2709.4**	**2975.0**

Table 109 Adaptor Oligo Mix dilution - used on page 68

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Nuclease-free water	2.5 µL	42.5	63.8	85.0	106.3	127.5	143.5	170.0	191.3	212.5	233.8	255.0	276.3
XT HS2 RNA Adaptor Oligo Mix* (tube with green cap)	5 µL	85.0	127.5	170.0	212.5	255.0	287.0	340.0	382.5	425.0	467.5	510.0	552.5
Total Volume	**7.5 µL**	**127.5**	**191.3**	**255.0**	**318.8**	**382.5**	**430.5**	**510.0**	**573.8**	**637.5**	**701.3**	**765.0**	**828.8**

* You can substitute the XT HS2 RNA Adaptor Oligo Mix with the XT HS2 DNA Adaptor Oligo Mix from the SureSelect XT HS2 DNA Library Preparation Kit for ILM. If you do so, prepare a 1:5 dilution of the XT HS2 DNA Adaptor Oligo Mix using low TE buffer before adding the Adaptor Oligo Mix to the master mix.

Table 110 Master mix source plate for 01 LibraryPrep_XT_HS2_ILM protocol - used on page 69

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Agilent Deep Well Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
End Repair-dA Tailing master mix	Column 4 (A4-H4)	31.0	52.0	73.0	94.0	115.0	136.0	158.8	182.0	205.8	230.0	254.8	280.0
Ligation master mix	Column 5 (A5-H5)	36.0	62.0	88.0	114.0	140.0	166.0	195.7	226.5	258.3	291.1	325.1	360.0
Adaptor Oligo Mix dilution	Column 6 (A6-H6)	15.0	22.5	30.0	37.5	45.0	52.5	60.6	68.8	76.9	85.0	93.1	101.3

Pre-Capture PCR

Table 111 Pre-Capture PCR Master Mix - used on page 76

Reagent	Volume for 1 Library	Volume (µL) Based on Number of Columns of Samples (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
5× Herculase II Buffer with dNTPs (tube with clear cap)	10 µL	170.0	255.0	340.0	425.0	510.0	574.0	656.0	738.0	820.0	902.0	984.0	1066
Herculase II Fusion DNA Polymerase (tube with red cap)	1 µL	17.0	25.5	34.0	42.5	51.0	57.4	65.6	73.8	82.0	90.2	98.4	106.6
Total Volume	**11 µL**	**187.0**	**280.5**	**374.0**	**467.5**	**561.0**	**631.4**	**721.6**	**811.8**	**902.0**	**992.2**	**1082.4**	**1172.6**

Table 112 Master mix source plate for 03 Pre-CapPCR_XT_HS2_ILM protocol - used on page 76

Master Mix Solution	Position on Source Plate	Volume (µL) of Master Mix added per Well of Selected Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
Pre-Capture PCR Master Mix	Column 1 (A1-H1)	22	33	44	55	66	77	88	99	110	121	132	143

Table 113Sample Buffer source plate for protocol 05 TS_D1000 - used on page 83

Solution	Position on Source Plate	Volume (µL) of Sample Buffer added per Well of Processing Source Plate Based on Number of Columns in the Run (1 to 12 Columns)											
		1	2	3	4	5	6	7	8	9	10	11	12
D1000 Sample Buffer	Column 2 (A2-H2)	11.0	17.0	23.0	29.0	35.0	41.0	47.0	53.0	59.0	65.0	71.0	77.0

Hybridization

Table 114 Block master mix - used on page 105

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

Table 115 Probe Hybridization master mix for Probes <3 Mb, 8 rows of wells - used on page 106

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

Table 116 Probe Hybridization master mix for Probes ≥3 Mb, 8 rows of wells - used on page 106

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

Table 117 Probe Hybridization master mix for Probes <3 Mb, single row of wells - used on page 107

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

Table 118 Probe Hybridization master mix for Probes ≥3 Mb, single row of wells - used on page 107

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

Table 119 Master mix source plate for 07 Hyb_XT_HS2_ILM protocol - used on page 108

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

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

Hybrid Capture and Washing

Table 120 Magnetic bead washing mixture - used on page 114

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

Table 121 Resuspension volumes for washed bead suspension - used on page 114

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

Post-Capture PCR

Table 122 Post-Capture PCR master mix - used on page 124

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

Table 123 Master mix source plate for 10 Post-CapPCR_XT_HS2_ILM protocol - used on page 124

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

Quick Reference Tables for Other Reagent Volumes

This section contains tables that summarize the cDNA 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 124 Total RNA Input by Grade

RNA grade	DV200	Recommended input amount	Minimum input amount
Intact RNA (from fresh or frozen samples)	>50%	200 ng	10 ng
Good FFPE RNA	>50%	200 ng	10 ng
Poor FFPE RNA	20% to 50%	200 ng	50 ng*
Inapplicable FFPE RNA	<20%	Not recommended for further processing	

* For optimal results, prepare libraries from poor-grade FFPE RNA samples using a minimum of 50 ng input RNA. Libraries may be prepared from 10–50 ng poor-grade FFPE RNA with potential negative impacts on yield or NGS performance

Table 125 XT HS2 Index Primer Pairs Volume on Primer Plate

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

Table 126 AMPure XP Bead Volumes for AMPure XP Protocols

Protocol	Volume of AMPure Beads per Well*
00d Cleanup_cDNA_XT_HS2_ILM	105 µL
02 Cleanup_LibPrep_XT_HS2_ILM	80 µL
04a Cleanup_Pre-CapPCR_SP_XT_HS2_ILM	50 µL
04b Cleanup_Pre-CapPCR_MP_XT_HS2_ILM	50 µL
06c PoolingConcentration_XT_HS2_ILM	180 µL
11 Cleanup_Post-CapPCR_XT_HS2_ILM	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 127 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 deep well reservoir	30 mL

Troubleshooting Guide

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 86**. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield of pre-capture libraries.
- ✔ For whole-genome sequencing (not specifically supported by this protocol), samples with an adaptor-dimer peak must be subjected to an additional round of SPRI-purification. To complete, first dilute the sample to 50 µL with nuclease free water, then run the AMPureXP_XT_HS2_ILM (Pre-Capture PCR) protocol.

If yield of post-capture libraries is low

- ✔ PCR cycle number may require optimization. Repeat library preparation and target enrichment for the sample, increasing the post-capture PCR cycle number by 1 to 2 cycles.
- ✔ The RNA Probe used for hybridization may have been compromised. Verify the expiration date on the Probe vial or Certificate of Analysis. Adhere to the recommended storage and handling conditions. Ensure that the Probe Hybridization Mix is kept on ice until it is dispensed into the master mix source plate, as directed on **page 106**, and that solutions containing the Probe are not held at room temperature for extended periods.

If post-capture library fragment size is different than expected in electropherograms

- ✔ DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. When preparing the AMPure XP bead plate for post-capture purification, mix the beads until the suspension appears homogeneous and consistent in color before adding the bead suspension to the shallow well reservoir. After preparation of the AMPure XP bead plate, seal the plate and store at 4°C until needed.

If low % on-target is observed in library sequencing results

- ✔ Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a thermal cycler in close proximity to the Bravo NGS Bravo to retain the 65°C sample temperature during transfer step (**step 16** on **page 112**).

If low uniformity of coverage with high AT-dropout is observed in library sequencing results

- ✔ High AT-dropout may indicate that hybridization conditions are too stringent to obtain the desired level of coverage for AT-rich targets.
  - For libraries target-enriched using the SureSelect XT HS Human All Exon V8 Probe or SureSelect XT HS PreCap Human All Exon V8 Probe and the hybridization program in **Table 53** on page 104 (including segment with one-hour incubation at 65°C), repeat target enrichment using the hybridization program in **Table 54** on page 104 (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 54** on page 104).

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In This Book

This guide contains information to run the SureSelect XT HS2 protocol for RNA, with optional pre-capture pooling, using automation protocols provided with the Agilent NGS Bravo Option A.

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