SureSelect XT HS2 mRNA Library Preparation System

Automated using Agilent NGS Workstation Option B

Poly-A Selection and Strand-Specific mRNA Seq Library Preparation for the Illumina Platform

Protocol

Version A1, December 2022

SureSelect platform manufactured with Agilent SurePrint technology.

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

Notices

Manual Part Number

G9995-90010

Edition

Version A1, December 2022

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Acknowledgment

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In this Guide...

This guide provides an optimized protocol for preparation of Illumina paired-end multiplexed mRNA sequencing libraries. Sample processing steps are automated using the Agilent NGS Workstation Option B.

1 Before You Begin

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

2 Using the Agilent NGS Workstation for SureSelect Library Preparation

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

3 Analysis of Total RNA Samples

This chapter describes the assessment of RNA quality and preparation of the RNA samples.

4 Preparation of AMPure XP Bead Plates

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

5 Selection of Poly-A mRNA and Conversion to cDNA

This chapter describes the steps for enriching the RNA samples for poly-A mRNA, fragmenting the RNA, and converting the RNA fragments to strand-specific cDNA prior to sequencing library preparation.

6 Library Preparation

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

7 Guidelines for Multiplexed Sequencing

This chapter provides instructions to pool the indexed, molecular barcoded samples and provides guidelines for multiplexed sequencing.

8 Reference

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

What’s New in Version A1

- Correction to the Bravo deck location for the empty processing plate during the Library Prep runsets (see **Table 42** on page 71).
- Correction to the number of tip boxes to load inin the BenchCel when processing 3 columns in the LibraryPrep_LILQ_XT_HS2_ILM runset (see **Table 44** on page 72).

Content

1 Before You Begin

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

NOTE This protocol describes automated RNA sample processing using the SureSelect XT HS2 mRNA Library Preparation System for cDNA synthesis and library preparation on the Agilent NGS Workstation Option B. For non-automated sample processing procedures see publication G9995-90000.

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 Workstation to allow rapid and efficient plate transfer.
- If your thermal cycler is compatible with the use of compression pads, add a compression pad whenever you load a plate that was sealed with the PlateLoc thermal microplate sealer. The pad improves contact between the plate and the heated lid of the thermal cycler.
- Prepare and load the Agilent NGS Workstation as detailed in each of the protocol steps before initiating each automated protocol run. When loading plates in the workstation's Labware MiniHub, always place plates in the orientation shown in **Figure 3** on page 48.
- 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 and ribonuclease contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow.
  2. Maintain clean work areas. Clean the surfaces that pose the highest risk of contamination daily using a 10% bleach solution, or equivalent.
  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 4°C or –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.

Safety Notes

CAUTION

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

Materials Required

Materials required to complete the SureSelect XT HS2 mRNA protocol are listed in the tables in this section. Select the preferred SureSelect XT HS2 mRNA Reagent Kit format from **Table 1**, and refer to **Table 2** through **Table 4** for additional materials needed to complete the protocols.

Table 1 SureSelect XT HS2 mRNA Library Preparation Kit Varieties

Description	Kit Part Numbers
SureSelect XT HS2 mRNA Library Preparation Kit, 96 reactions	G9997A (with Index Pairs 1–96) G9997B (with Index Pairs 97–192) G9997C (with Index Pairs 193–288) G9997D (with Index Pairs 289–384)
SureSelect XT HS2 mRNA Library Preparation Kit with AMPure® XP Beads*, 96 reactions	G9998A (with Index Pairs 1–96) G9998B (with Index Pairs 97–192) G9998C (with Index Pairs 193–288) G9998D (with Index Pairs 289–384)

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

Table 2 Required Reagents

Description	Vendor and part number
AMPure XP Kit* 60 mL 450 mL	Beckman Coulter Genomics p/n A63881 p/n A63882
1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930
QPCR Human Reference Total RNA –for use as control input RNA (optional)	Agilent p/n 750500
Tween 20 –for use as an additive if storing libraries prior sequencing (optional)	Sigma-Aldrich p/n P9416-50ML

* Separate purchase **not** required when using the SureSelect XT HS2 mRNA Reagent Kits that include SureSelect RNA AMPure® XP Beads (Agilent p/n G9998A, G9998B, G9998C, or G9998D).

Table 3 Required Equipment

Description	Vendor and Part Number
Agilent NGS Workstation Option B Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5522A (VWorks software version 13.1.0.1366 OR Agilent p/n G5574AA (VWorks software version 13.1.0.1366)
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Thermal cycler and accessories (including compression pads, if compatible)	Various suppliers *Important*: Not all PCR plate types are supported for use in the VWorks automation protocols for the Agilent NGS Workstation. Select a thermal cycler that is compatible with one of the supported PCR plate types. See supported plate types in the listing below.
PCR plates compatible with the Agilent NGS Workstation and associated VWorks automation protocols	Only the following PCR plates are supported: • 96 ABI PCR half-skirted plates (MicroAmp Optical plates), Thermo Fisher Scientific p/n N8010560 • 96 Agilent semi-skirted PCR plate, Agilent p/n 401334 • 96 Eppendorf Twin.tec half-skirted PCR plates, Eppendorf p/n 951020303 • 96 Eppendorf Twin.tec PCR plates (full-skirted), Eppendorf p/n 951020401 • 96 Armadillo PCR plates (full-skirted), Thermo Fisher Scientific p/n AB2396
Armadillo PCR plates, 96-wells (full-skirted)	Thermo Fisher Scientific p/n AB2396
Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 19 mm height – used when workstation setup calls for **Agilent Shallow Well Reservoir**	Agilent p/n 201254-100
Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 44 mm height – used when workstation setup calls for **Agilent Deep Well Reservoir**	Agilent p/n 201244-100
Agilent Storage/Reaction Microplates, 96 wells, 1 mL/round well – used when workstation setup calls for **Agilent Deep Well Plate** or **Agilent DW Plate**	Agilent p/n 203426-100
Agilent Storage/Reaction Microplates, 96 wells, 2 mL/square well – used when workstation setup calls for **Waste Plate (Agilent 2 mL Square Well)**	Agilent p/n 201240-100
Nucleic acid surface decontamination wipes	DNA Away Surface Decontaminant Wipes, Thermo Scientific p/n 7008, or equivalent
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
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

Table 3 Required Equipment (continued)

Description	Vendor and Part Number
Vortex mixer	general laboratory supplier
Ice bucket	general laboratory supplier
Powder-free gloves	general laboratory supplier

Table 4 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
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
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

2 Using the Agilent NGS Workstation for SureSelect Library Preparation

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

About the Agilent NGS Workstation

CAUTION Before you begin, make sure that you have read and understand operating, maintenance and safety instructions for using the Bravo platform and additional devices included with the workstation. Refer to the user guides listed in **Table 5**.

Review the user guides listed in **Table 5** (available at www.agilent.com) to become familiar with the general features and operation of the Agilent NGS Workstation Option B components. Instructions for using the Bravo platform and other workstation components specifically for the SureSelect XT HS2 mRNA Library Preparation workflow are detailed in this user guide.

Table 5 Agilent NGS Workstation components User Guide reference information

Device	User Guide part number
Bravo Platform	SD-V1000376 (formerly G5562-90000)
VWorks Software v13.1	G5415-90068
BenchCel Microplate Handler	G5580-90000
Labware MiniHub	G5584-90001
PlateLoc Thermal Microplate Sealer	G5585-90010

About the Bravo Platform

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

Bravo Platform Deck

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

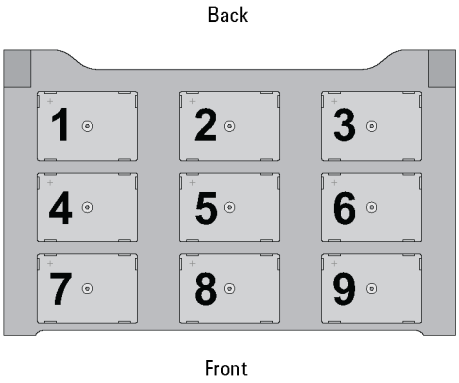


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

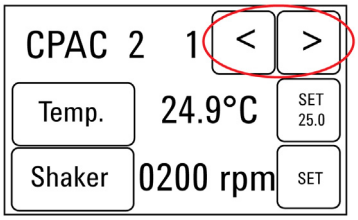
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

Bravo deck heat block temperatures may be changed using the Inheco Multi TEC Control device touchscreen as described in the steps below. See **Table 6** for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

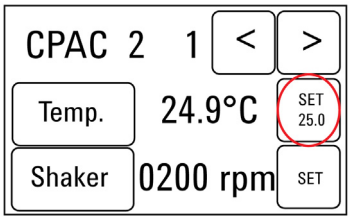
Table 6 Inheco Multi TEC Control touchscreen designations

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

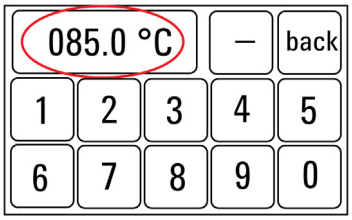
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



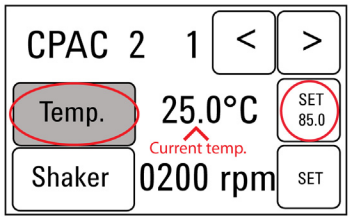
2 To set the temperature of the selected block, press the SET button.



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

NOTE

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

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

Logging in to the VWorks software

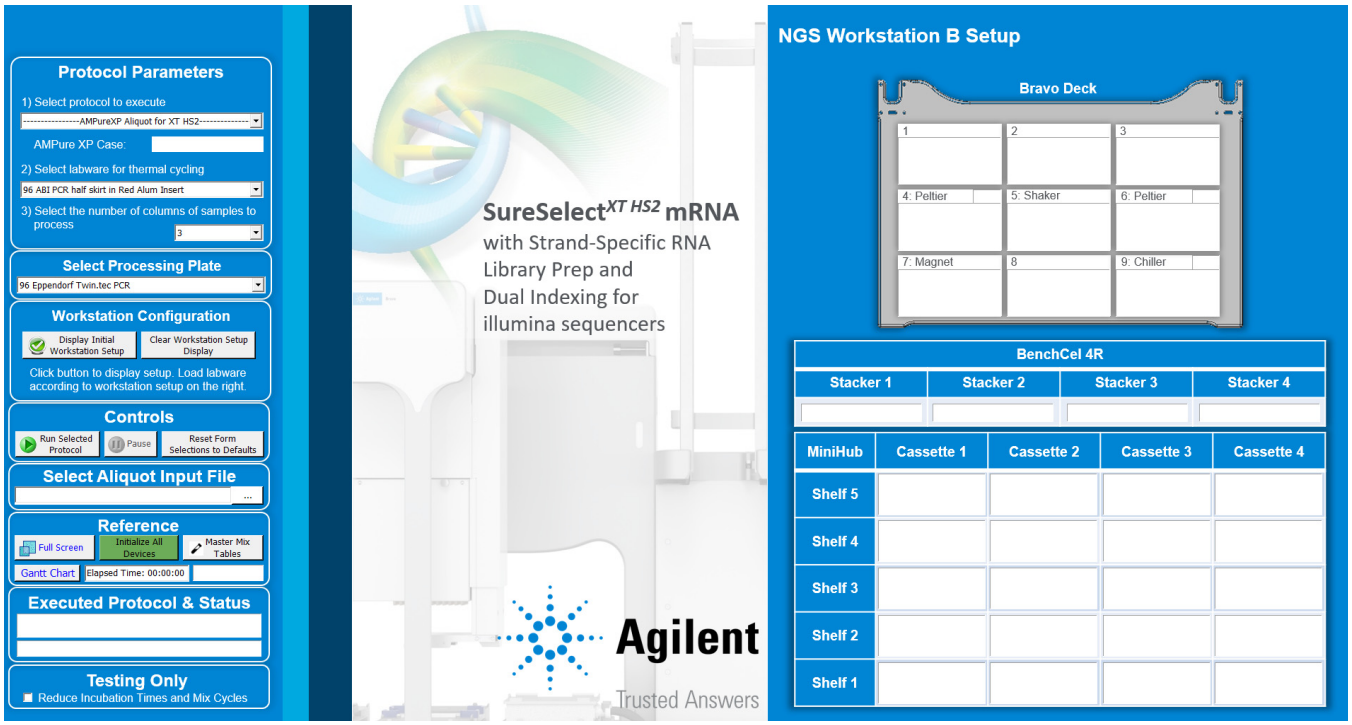
1. Double-click the VWorks icon or the XT HS2 mRNA VWorks Form shortcut on the Windows desktop to start the VWorks software.
2. If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
3. In the User Authentication dialog, type your VWorks user name and password, and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

VWorks protocol and runset files

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

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

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



1 Open the form using the shortcut (shown below) on your desktop, or by opening the file **XT_HS2_mRNA_ILM_v.Bx.x.x.VWForm** in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_mRNA_ILM_v.Bx.x.x\Forms** (where x.x.x is the version number).

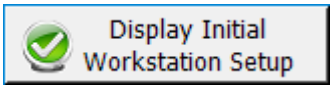
2 Verify that the Processing Plate selection is set to the correct plate type.

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).

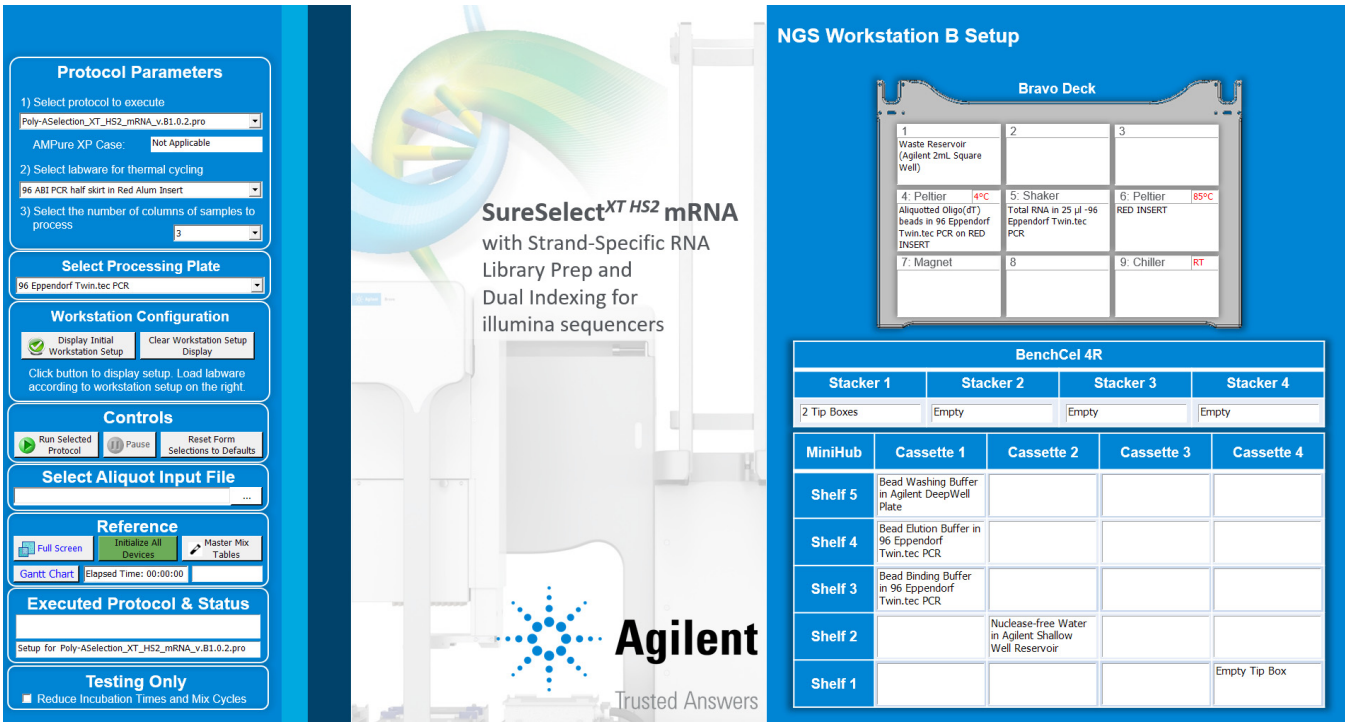
CAUTION Indicating the correct processing plate type that will be used in the assay is critical for accurate pipetting and to avoid damage to the Bravo instrument.

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

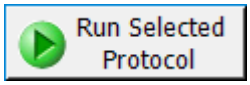
4 Once all run parameters have been specified on the form, click **Display Initial Workstation Setup**.



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



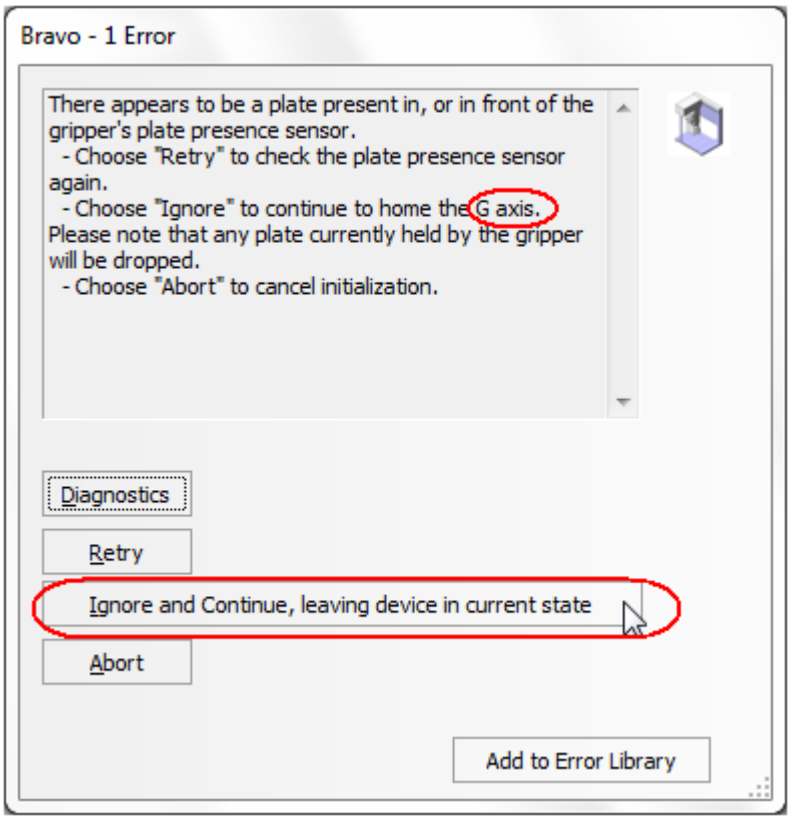
6 After verifying that the NGS Workstation has been set up correctly, click **Run Selected Protocol**.



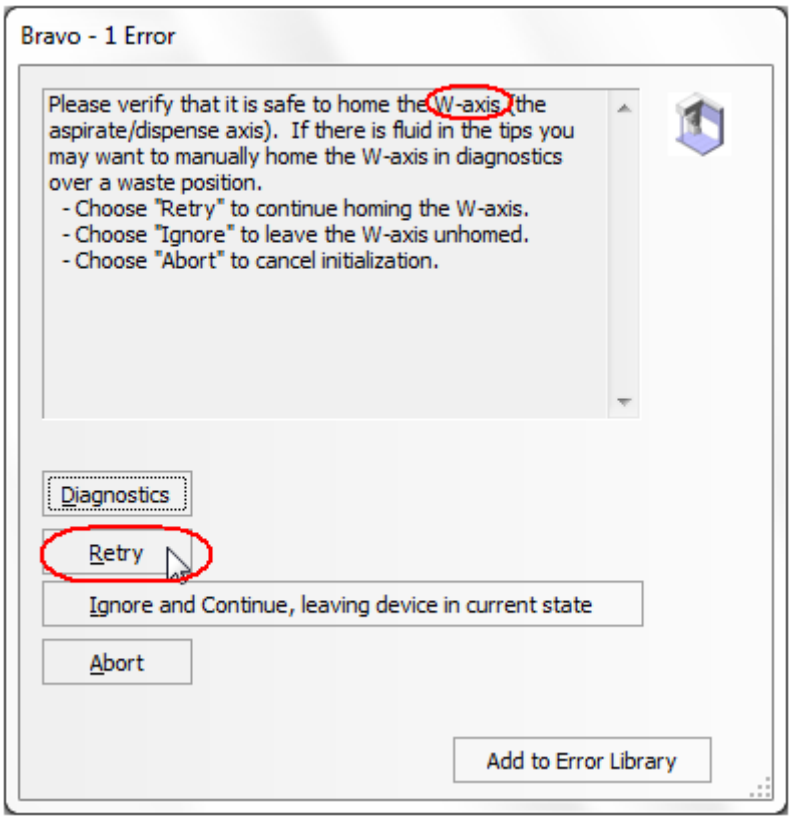
Error messages encountered at start of run

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

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



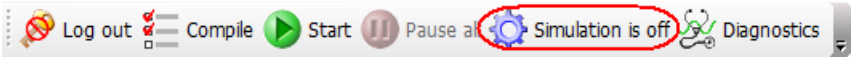
2 If you encounter the W-axis error message shown below, select **Retry**.



Verifying the Simulation setting

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

1 Verify that Simulation is off is displayed on the status indicator (accessible by clicking **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 SureSelect_XT_HS2_mRNA_ILM VWorks form, click the **Full Screen** button to exit full screen mode. If the toolbar is still not visible, right-click on the form and then select **Control Toolbar** from the menu.

Overview of the Workflow

The SureSelect XT HS2 mRNA workflow for the preparation of NGS-ready libraries is summarized in **Figure 2**.

See **Table 7** for a summary of the VWorks protocols used during the workflow. Then, see **Selection of Poly-A mRNA and Conversion to cDNA**, **Library Preparation**, and **Guidelines for Multiplexed Sequencing** chapters for complete instructions for use of the VWorks protocols for sample processing.

The SureSelect XT HS2 mRNA library preparation protocol is compatible with total RNA prepared from fresh or fresh frozen samples.

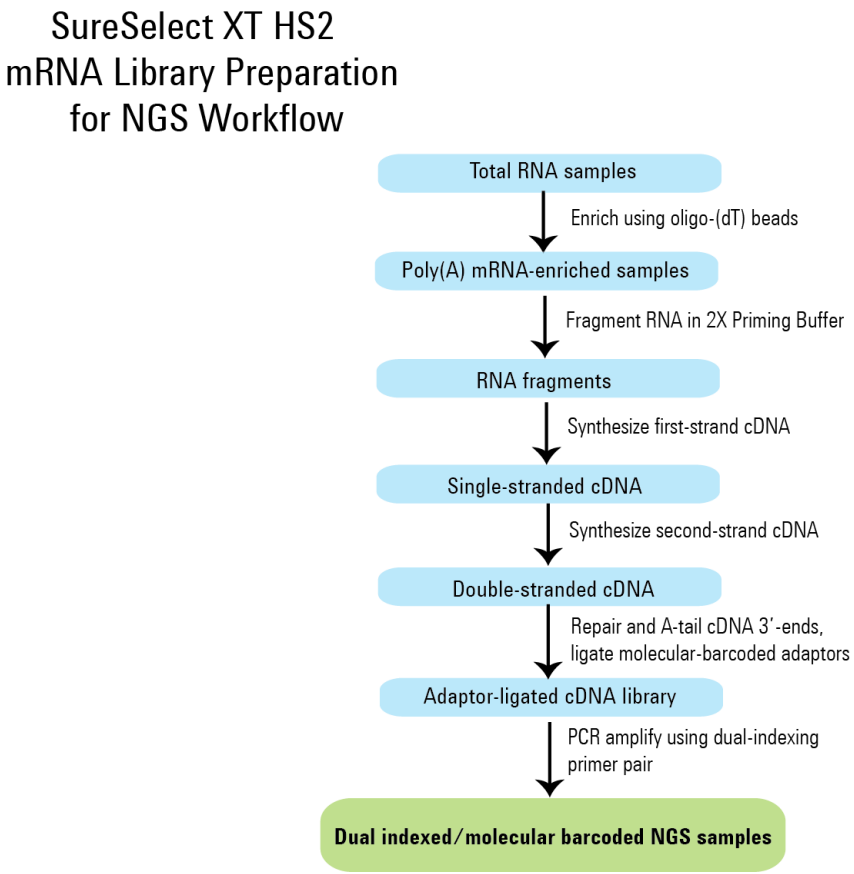


Figure 2 Overall sequencing sample preparation workflow

Automation Protocols used in the Workflow

Table 7 Overview of VWorks protocols and runsets

Workflow Step	Substep	VWorks Protocols Used for Agilent NGS Workstation automation
AMPure XP Bead Aliquoting	Aliquot AMPure XP beads for use in the Second-Strand Synthesis runset	AMPureXP_Aliquot (Case Second-Strand)
	Aliquot AMPure XP beads for use in the Library Prep runset	AMPureXP_Aliquot (Case Library Prep)
	Aliquot AMPure XP beads for use in the Library Prep runset for low input (<100 ng) or lower-quality (RIN 6–8) libraries	AMPureXP_Aliquot (Case Low Input or Quality)
	Aliquot AMPure XP Beads for use in the PCR purification protocol	AMPureXP_Aliquot (Case PCR)
Poly(A) Selection	Enrich RNA samples for poly(A) mRNA	Poly-ASelection_XT_HS2_mRNA
RNA Preparation and cDNA Conversion	Fragment mRNA samples	Fragmentation_XT_HS2_mRNA
	Synthesize first-strand cDNA	FirstStrandcDNA_XT_HS2_mRNA
	Synthesize and purify second-strand cDNA	SecondStrand_XT_HS2_mRNA
Library Preparation	Prepare duplex, molecular-barcoded cDNA libraries	Runset LibraryPrep_XT_HS2_ILM *or* Runset LibraryPrep_LILQ_XT_HS2_ILM (for low input and lower-quality libraries)
Amplification	Amplify indexed cDNA libraries with unique dual indexing primer pair	PCR_XT_HS2_ILM
	Purify indexed cDNA libraries using AMPure XP beads	AMPureXP_XT_HS2_ILM (Case PCR)
	Analyze indexed cDNA libraries using Agilent TapeStation platform	TS_D1000
Pooling	Pool indexed cDNA libraries for multiplexed sequencing	Aliquot_mRNA_Libraries

Experimental Setup Considerations for Automated Runs

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

Table 8 Columns to Samples Equivalency

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

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

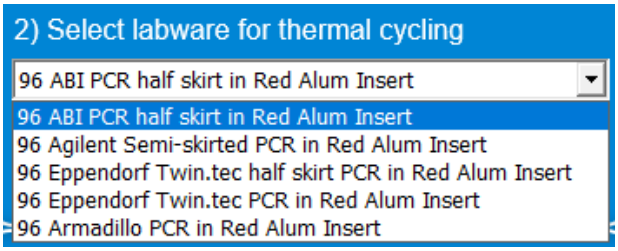
- The Agilent NGS Workstation processes samples column-wise beginning at column 1. The samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.

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 Agilent NGS Workstation to allow rapid and efficient plate transfer.
- Several workflow steps require that the sample plate be sealed using the PlateLoc thermal microplate sealer included with the Agilent NGS Workstation, and then centrifuged to collect any dispersed liquid. To maximize efficiency, locate the centrifuge in close proximity to the Agilent NGS Workstation.

PCR Plate Type Considerations

Automation protocols include several liquid-handling steps in which reagents are dispensed to PCR plates in preparation for transfer to a thermal cycler. For these steps, you must specify on the VWorks Form which PCR plate type will be used to allow correct configuration of the liquid handling components. Before you begin the automation protocol, make sure that you are using a supported PCR plate type. The PCR plate type to be used in the protocol is specified using the menu below. Vendor and part number information is provided for the supported plate types in **Table 9**.



CAUTION

The plates listed in **Table 9** 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 9**, even if they are compatible with your chosen thermal cycler.

Table 9 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 Analysis of Total RNA Samples

This chapter describes the assessment of RNA quality and preparation of the RNA samples.

The protocol is compatible with intact RNA prepared from fresh or fresh frozen samples. This protocol is not recommended for FFPE-derived RNA samples.

Step 1. Assess quality of total RNA

Before you begin, prepare total RNA from each sample in the run in nuclease-free water. The library preparation protocol requires 10 ng to 1 µg high-quality total RNA. A minimum RNA input of 50 ng is required for samples with reduced RNA integrity, as detailed below.

NOTE The protocol in this publication is suitable for intact RNA prepared from fresh or fresh frozen samples. This protocol is not recommended for FFPE-derived RNA samples.

Consider preparing an additional sequencing library in parallel, using a high-quality control RNA sample, such as Agilent's QPCR Human Reference Total RNA (p/n 750500). Use of this control is especially recommended during the first run of the protocol, to verify that all protocol steps are being successfully performed. Routine use of this control is helpful for any required troubleshooting, in order to differentiate any performance issues related to RNA input from other factors.

1 Prepare total RNA for each sample in the run in nuclease-free water.

2 Determine the RNA concentration using a small volume spectrophotometer. Verify that the 260/280 and 260/230 absorbance ratio values for the sample are both approximately 1.8 to 2.0. A significant deviation from ratios of 2.0 indicates that the sample may require further purification before use in NGS library preparation.

3 Analyze RNA integrity by determining the RNA Integrity Number (RIN), or equivalent, using one of the RNA qualification platforms listed in **Table 4** on page 15. The RIN/RIN^e/RQN quality scores reported by these Agilent platforms are equivalent measures of RNA quality. Select the specific RNA assay for your platform based on the concentration determined in **step 2**.

   For optimal performance, total RNA samples should have RIN>8. For samples with RIN>8, the amount of total RNA needed for the library preparation protocol is 10 ng to 1 µg.

   Samples with RIN of 6 to 8 may be used in the protocol, using a minimum RNA input of 50 ng.

   Samples with RIN<6 are not suitable for use in this protocol; instead consider Agilent's SureSelect XT HS2 RNA system for use with the Agilent NGS Workstation (see publication G9989-90010 at www.agilent.com).

Step 2. Prepare the plate of total RNA samples

NOTE The VWorks form for SureSelect XT HS2 mRNA library preparation offers two different Library Preparation runsets (standard or low-input/low-quality).The RNA input quantity and RNA quality determine which runset is appropriate for your run. You may want to select input quantities that ensure that all samples can be processed together in the same Library Preparation runset. Refer to **Table 10** for further details.

1 In the wells of a fresh processing plate (Eppendorf twin.tec or Armadillo plate), dilute the appropriate quantity of each RNA sample with nuclease-free water to final volume of 25 µL. Consult **Table 10** for the appropriate RNA quantity for your sample based on the RIN.

Table 10 also indicates which Library Preparation runset is recommended for the samples based on input quantity and RIN. The Library Preparation step is performed in **"Step 1. Prepare adaptor-ligated libraries"** on page 66.

Table 10 RNA input quantity and recommended Library Preparation runset

RIN of RNA sample	RNA input quantity	Library Preparation runset
>8	100 ng to 1 µg	LibraryPrep_XT_HS2_ILM
	10 ng to 100 ng	LibraryPrep_LILQ_XT_HS2_ILM
6–8	50 ng to 1 µg	LibraryPrep_LILQ_XT_HS2_ILM
<6	Not recommended for use	—

2 Seal the total RNA sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep on ice until needed in **step 2** on **page 48**.

4 Preparation of AMPure XP Bead Plates

This chapter provides instructions on preparing all of the plates of AMPure XP beads that are needed throughout the entire workflow. Each plate of AMPure XP beads is prepared using a separate automation protocol available in the XT HS2 VWorks form. For an overview of the SureSelect XT HS2 mRNA workflow, see **Figure 2** on page 26.

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 second-strand cDNA synthesis

The SecondStrand_XT_HS2_RNA protocol requires a bead plate containing 105 µL of beads in each well. Use the AMPureXP_Aliquot (Second-Strand) protocol to prepare the bead plate needed for second-strand cDNA synthesis.

Prepare the workstation and reagents for the AMPureXP_Aliquot (Second-Strand) protocol

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.
   a. Verify that the AMPure XP bead suspension is at room temperature.
   b. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
   c. Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the SecondStrand_XT_HS2_RNA protocol (each column accommodates 8 DNA libraries).

Load the Agilent NGS Workstation

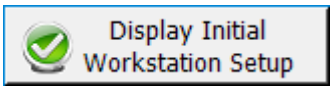
1. Load the Bravo deck according to **Table 11**.

Table 11 Initial Bravo deck configuration for AMPureXP_Aliquot (Second-Strand) protocol

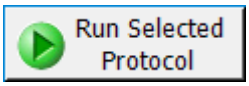
Location	Content
2	New tip box
5	Empty Agilent Deep Well Plate
6	Reservoir of AMPure XP bead suspension prepared in **step 3**
8	Empty tip box

Run VWorks protocol AMPureXP_Aliquot (Second-Strand)

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



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



   Running the AMPureXP_Aliquot (Second-Strand) protocol takes approximately 5 minutes. The protocol directs the Bravo NGS Workstation to aliquot 105 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent Deep Well plate.
6. When the protocol is complete, remove the Agilent Deep Well plate containing the AMPure XP bead suspension from position 5 of the Bravo deck.
7. 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 SecondStrand_XT_HS2_RNA protocol (refer to **Table 33** on page 62). 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 plates to be used for library preparation

The SureSelect XT HS2 mRNA VWorks form offers two different runsets for the library preparation step of the workflow: the LibraryPrep_XT_HS2_ILM runset and the LibraryPrep_LILQ_XT_HS2_ILM runset. The LibraryPrep_LILQ_XT_HS2_ILM runset is specifically for low-input or low-quality samples. See **Table 10** on page 33 for guidance on selecting the appropriate library preparation runset based on the quality and quantity of the total RNA samples.

Both library preparation runsets require a bead plate containing 80 µL of beads in each well. Use the AMPureXP_Aliquot (Library Prep) protocol to prepare the this bead plate. See **"Run the AMPureXP_Aliquot (Library Prep) protocol"** on page 38 for instructions on running the AMPureXP_Aliquot (Library Prep) protocol.

The LibraryPrep_LILQ_XT_HS2_ILM runset requires an additional bead plate that contains 60 µL of beads in each well. Use the AMPureXP_Aliquot (Low Input or Quality) protocol to prepare the this bead plate. See **"Run the AMPureXP_Aliquot (Low Input or Quality) protocol (if required)"** on page 40 for instructions on running the AMPureXP_Aliquot (Low Input or Quality) protocol.

Run the AMPureXP_Aliquot (Library Prep) protocol

This protocol prepares the bead plate containing 80 µL of beads in each well. Both runset options for library preparation require this bead plate.

Prepare the workstation and reagents for the AMPureXP_Aliquot (Library Prep) protocol

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.
   a. Verify that the AMPure XP bead suspension is at room temperature.
   b. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
   c. Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the LibraryPrep_XT_HS2_ILM protocol (each column accommodates 8 cDNA samples).

Load the Agilent NGS Workstation

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

Table 12 Initial Bravo deck configuration for AMPureXP_Aliquot (Library Prep) protocol

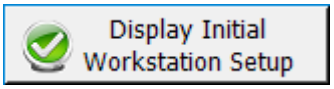
Location	Content
2	New tip box
5	Empty Agilent Deep Well plate
6	Reservoir of AMPure XP bead suspension prepared in **step 3**
8	Empty tip box

Run VWorks protocol AMPureXP_Aliquot (Library Prep)

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

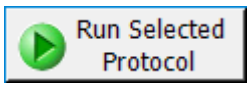
6 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

7 Click **Display Initial Workstation Setup**.



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

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



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

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

11 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the Library Preparation protocol (refer to **Table 41** on page 71). 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.

Run the AMPureXP_Aliquot (Low Input or Quality) protocol (if required)

This protocol prepares the bead plate containing 60 µL of beads in each well. Only the library preparation runset for low input or low quality samples (LibraryPrep_LILQ_XT_HS2_ILM) requires this bead plate. If you do not plan to run the LibraryPrep_LILQ_XT_HS2_ILM runset, you do not need to prepare this bead place and may proceed directly to **"Step 3. Prepare the bead plate to be used for PCR Purification"** on page 42.

Prepare the workstation and reagents for the AMPureXP_Aliquot (Low Input or Quality) protocol

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.
   a. Verify that the AMPure XP bead suspension is at room temperature.
   b. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
   c. Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the LibraryPrep_XT_HS2_ILM protocol (each column accommodates 8 cDNA samples).

Load the Agilent NGS Workstation

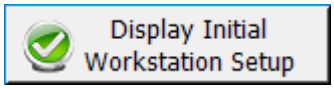
4. Load the Bravo deck according to **Table 12**.

Table 13 Initial Bravo deck configuration for AMPureXP_Aliquot (Low Input or Quality) protocol

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

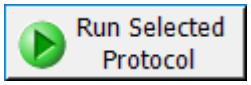
Run VWorks protocol AMPureXP_Aliquot (Low Input or Quality)

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



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

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



Running the AMPureXP_Aliquot (Low Input or Quality) protocol takes approximately 5 minutes. The protocol directs the Bravo NGS Workstation to aliquot 60 µL of beads from the Agilent shallow well reservoir into the wells of the Agilent Deep Well plate.

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

11 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the LibraryPrep_LILQ_XT_HS2_ILM protocol (refer to **Table 41** on page 71). 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 PCR Purification

The AMPureXP_XT_HS2_ILM (PCR) protocol requires a bead plate containing 50 µL of beads in each well. Use the AMPureXP_Aliquot (PCR) protocol to prepare the bead plate needed for purification of the PCR products.

Prepare the workstation and reagents for the AMPureXP_Aliquot (PCR) protocol

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Prepare an Agilent shallow well reservoir containing the AMPure XP bead suspension.
   a. Verify that the AMPure XP bead suspension is at room temperature.
   b. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
   c. Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the AMPureXP_XT_HS2_ILM (PCR) protocol (each column accommodates 8 indexed libraries).

Load the Agilent NGS Workstation

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

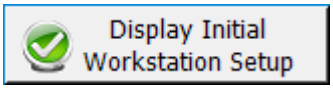
Table 14 Initial Bravo deck configuration for AMPureXP_Aliquot (PCR) protocol

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

Run VWorks protocol AMPureXP_Aliquot (PCR)

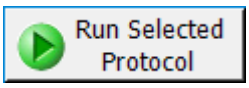
1. On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot (PCR)** protocol.
2. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

3 Click **Display Initial Workstation Setup**.



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

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



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

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

7 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the AMPureXP_XT_HS2_ILM (PCR) protocol (refer to **Table 53** 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.

5 Selection of Poly-A mRNA and Conversion to cDNA

This chapter describes the steps for enriching the RNA samples for poly-A mRNA, fragmenting the RNA, and converting the RNA fragments to strand-specific cDNA prior to sequencing library preparation.

The protocol is compatible with intact RNA prepared from fresh or fresh frozen samples. This protocol is not recommended for FFPE-derived RNA samples.

Steps in this section use the components listed in **Table 15** and **Table 16**. Before you begin the protocol, equilibrate the components in **Table 15** to room temperature and thaw the components in **Table 16** on ice. Mix each component as directed before use (see the *Where Used* column).

Table 15 Reagents brought to room temperature before use in the poly-A selection protocol

Kit Component	Storage Location	Where Used
Oligo(dT) Microparticles (tube with brown cap or bottle)	SureSelect Poly-A Selection Module (Pre PCR), 4°C	page 46
Bead Washing Buffer (bottle)	SureSelect Poly-A Selection Module (Pre PCR), 4°C	page 46
Bead Elution Buffer (tube with green cap or bottle)	SureSelect Poly-A Selection Module (Pre PCR), 4°C	page 46
Bead Binding Buffer (tube with purple cap or bottle)	SureSelect Poly-A Selection Module (Pre PCR), 4°C	page 46

Table 16 Reagents thawed and held on ice before use in fragmentation and cDNA synthesis protocols

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 51
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 56
Second Strand Enzyme Mix (tube with blue cap or bottle)	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	page 60
Second Strand Oligo Mix (tube with yellow cap)	SureSelect cDNA Module (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	page 60

* 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. Select poly-A mRNA from total RNA

This step uses automation protocol **Poly-ASelection_XT_HS2_mRNA**.

In this step, the Bravo NGS Workstation executes the steps for poly-A RNA selection from the total RNA samples using two serial rounds of binding to oligo(dT) magnetic particles, followed by washing of the bead-bound mRNA and elution of the mRNA samples.

Prepare the workstation for protocol Poly-ASelection_XT_HS2_mRNA

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 19. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Pre-set the temperature of Bravo deck position 6 to 85°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 red PCR plate inserts at Bravo deck positions 4 and 6.

Prepare the reagent plates

The automated protocol for poly-A selection requires separate 96-well plates of aliquoted reagents for the Oligo(dT) Microparticles, Bead Binding Buffer, Bead Washing Buffer, and Bead Elution Buffer.

1. Prepare each of the four required reagent plates according to the volumes and plate types listed in **Table 17**. In each reagent plate, add reagent only to the wells only for the number of columns in your run.

   Prior to removing an aliquot of Oligo(dT) Microparticles, vortex until the suspension appears homogeneous and consistent in color. If bead aggregates are still present after vortexing, mix thoroughly by pipetting up and down until the suspension appears homogeneous.

Table 17 Reagent plates for protocol Poly-ASelection_XT_HS2_mRNA

Reagent	Volume of reagent per well	Plate type
Oligo(dT) Microparticles	26 µL	Processing plate (Eppendorf twin.tec or Armadillo plate)
Bead Binding Buffer	26 µL	Processing plate (Eppendorf twin.tec or Armadillo plate)
Bead Washing Buffer	370 µL	Agilent Deep Well plate
Bead Elution Buffer	26 µL	Processing plate (Eppendorf twin.tec or Armadillo plate)

2 Seal the reagent plates using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep the plate at room temperature.

3 Briefly centrifuge the plates to drive the well contents off the walls and plate seal and to eliminate air bubbles. Keep the plates at room temperature.

When centrifuging the Oligo(dT) Microparticles plate, make sure that the microparticles do not form pellets in the plate wells.

The presence of bubbles in the wells may cause inaccurate volume transfer by the Bravo liquid handling platform. Ensure that the plates are sealed and centrifuged prior to use in a run.

Prepare the water reservoir

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

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

At the end of the automation runset, retain this reservoir for use in the Second Strand cDNA Synthesis automation protocol.

Load the Agilent NGS Workstation

1 Load the Labware MiniHub according to **Table 18**, using the plate orientations shown in **Figure 3**. Unseal the reagent plates prior to loading.

Table 18 Initial MiniHub configuration for Poly-ASelection_XT_HS2_mRNA protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Bead Washing Buffer plate (Agilent Deep Well plate) from **step 1**	—	—	—
Shelf 4	Bead Elution Buffer plate (Eppendorf twin.tec or Armadillo plate) from **step 1**	—	—	—
Shelf 3	Bead Binding Buffer plate (Eppendorf twin.tec or Armadillo plate) from **step 1**	—	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 1**	—	—
Shelf 1 (Bottom)	—	—	—	Empty tip box

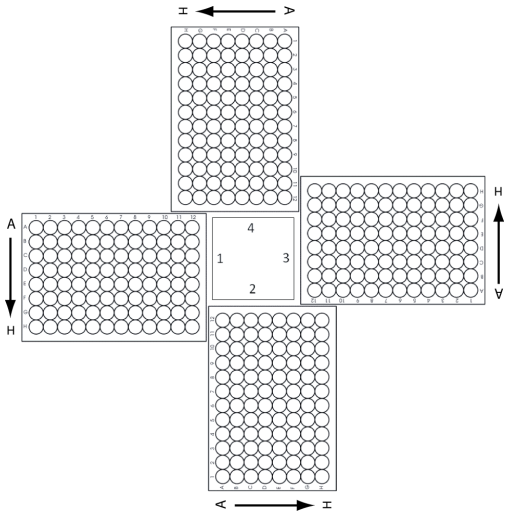


Figure 3 Agilent Labware MiniHub plate orientation

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

Table 19 Initial Bravo deck configuration for Poly-ASelection_XT_HS2_mRNA protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	Total RNA sample plate prepared on **page 33** (Eppendorf twin.tec or Armadillo plate), unsealed and seated in red insert
5	Olido(dT) Microparticle plate (Eppendorf twin.tec or Armadillo plate) from **step 1**, unsealed
6	Empty red insert

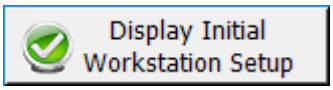
3 Load the BenchCel Microplate Handling Workstation according to **Table 20**.

Table 20 Initial BenchCel configuration for Poly-ASelection_XT_HS2_mRNA protocol

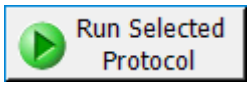
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	3 Tip boxes	—	—	—
12	5 Tip boxes	—	—	—

Run VWorks protocol Poly-ASelection_XT_HS2_mRNA

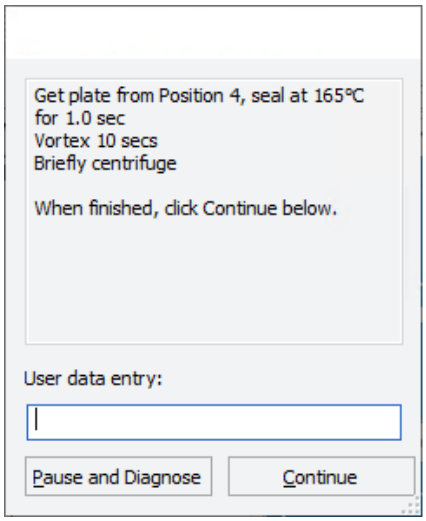
1. On the SureSelect setup form, under **Select protocol to execute**, select the **Poly-ASelection_XT_HS2_mRNA** protocol.
2. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
3. Verify that the **Processing Plate** selection is set to the correct plate type. See **page 22** for more information on using this segment of the form.
4. Click **Display Initial Workstation Setup**.



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



Running the Poly-ASelection_XT_HS2_mRNA protocol takes approximately 1 hour. At the completion of the protocol, the following prompt opens.



7. Remove the processing plate containing the mRNA samples 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.
8. Vortex the sealed plate for 10 seconds. Then, briefly centrifuge the plate (for 5–8 seconds) to drive the well contents off the walls and plate seal and to eliminate air bubbles.
9. In VWorks, click **Continue**.

Keep the sealed mRNA sample plate on ice or a cold block. Continue to **"Step 2. Fragment the mRNA samples"** on page 50.

Step 2. Fragment the mRNA samples

This step uses automation protocol **Fragmentation_XT_HS2_mRNA**.

The mRNA samples require fragmentation prior to cDNA synthesis. In this step, the Bravo NGS Workstation directs the preparation of the fragmentation reaction plate. At the end of the automated fragmentation protocol, the prepared plate is transferred to a thermal cycler for fragmentation of the mRNA samples at 94°C (see thermal cycling program in **Table 21**).

Prepare the workstation for protocol Fragmentation_XT_HS2_mRNA

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 19. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
5. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
6. Place red PCR plate inserts at Bravo deck positions 4 and 6.

Pre-program the thermal cycler

1. Pre-program a thermal cycler using the program in **Table 21**. Start the program, then immediately pause the program.

Table 21 Thermal cycler program for fragmentation of intact RNA samples*

Step	Temperature	Time
Step 1	94°C	4 minutes
Step 2	4°C	1 minutes
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 Fragmentation master mix source plate

1 Prepare the **Agilent Deep Well** source plate for the run as indicated in **Table 22**. 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 4**.

Table 22 Preparation of the Fragmentation master mix source plate for protocol Fragmentation_XT_HS2_RNA

Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
2X Priming Buffer (tube with purple cap)	Column 1 (A1-H1)	16.0 µL	27.0 µL	38.0 µL	49.0 µL	76.0 µL	145.0 µL

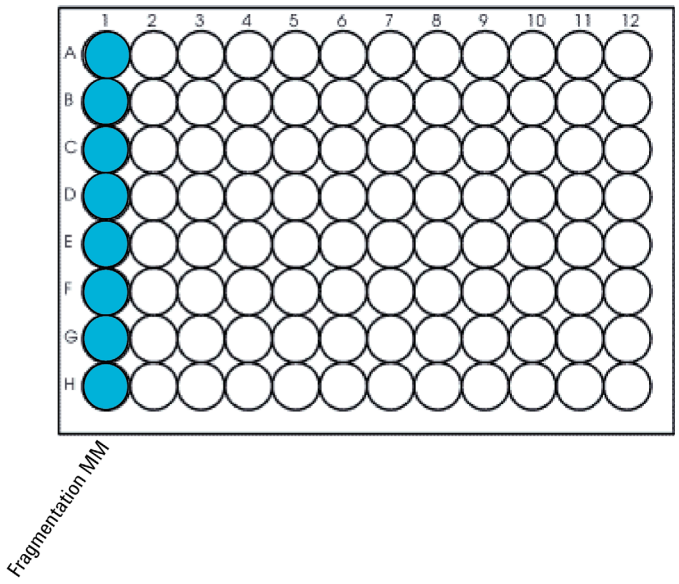


Figure 4 Configuration of the **Agilent Deep Well** source plate for protocol Fragmentation_XT_HS2_mRNA

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

3 Briefly centrifuge the plate 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 Agilent NGS Workstation

1 Load the Labware MiniHub according to **Table 18**, using the plate orientations shown in **Figure 3** on page 48.

Table 23 Initial MiniHub configuration for Fragmentation_XT_HS2_mRNA protocol

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

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

Table 24 Initial Bravo deck configuration for Fragmentation_XT_HS2_RNA protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	mRNA sample plate (Eppendorf twin.tec or Armadillo plate), unsealed and seated in red insert
6	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under Parameter 2)
9	Fragmentation master mix source plate, unsealed

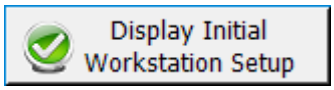
3 Load the BenchCel Microplate Handling Workstation according to **Table 20**.

Table 25 Initial BenchCel configuration for Fragmentation_XT_HS2_RNA protocol

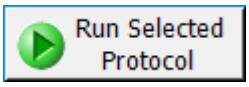
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Run VWorks protocol Fragmentation_XT_HS2_mRNA

1. On the SureSelect setup form, under **Select protocol to execute**, select the **Fragmentation_XT_HS2_mRNA** protocol.
2. Under **Select labware for thermal cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.
3. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
4. Verify that the **Processing Plate** selection is set to the correct plate type. See **page 22** for more information on using this segment of the form.
5. Click **Display Initial Workstation Setup**.

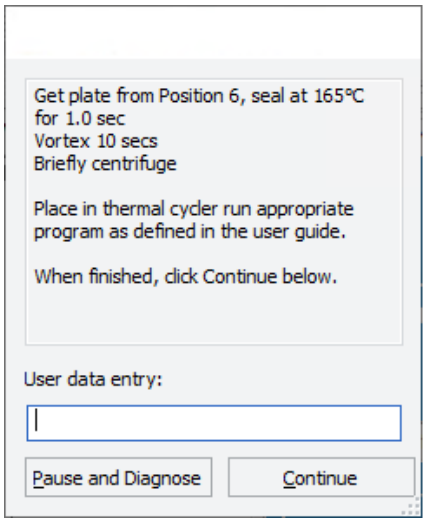


6. Verify that the NGS workstation has been set up as displayed on the right side of the form.
7. When verification is complete, click **Run Selected Protocol**.



Running the Fragmentation_XT_HS2_mRNA protocol takes approximately 10 minutes. Once complete, the samples are ready for fragmentation (performed in the pre-programmed thermal cycler). The samples are located in the PCR plate at position 6 of the Bravo deck.

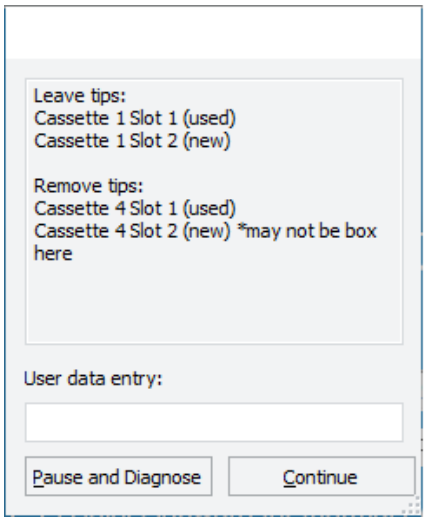
8. When you see the following prompt, remove the PCR plate from position 6 of the Bravo deck. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing setting of 165°C and 1.0 seconds.



9. Vortex the sealed plate at medium speed for 10 seconds, then briefly centrifuge the plate to drive the well contents off the walls and plate seal and to eliminate air bubbles.
10. Immediately place the sample plate in the thermal block. Close the lid and resume the thermal cycler program in **Table 21**. 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.

11 In VWorks, click **Continue**.

The following prompt opens.



12 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 FirstStrandcDNA_XT_HS2_mRNA protocol as described in **"Prepare the First Strand cDNA master mix source plate"** on page 56.

13 On the Labware MiniHub, leave the tip boxes in Cassette 1 in place. They will be used in the FirstStrandcDNA_XT_HS2_mRNA protocol as described in **"Load the Agilent NGS Workstation"** on page 57.

14 Once the thermal cycler program in **Table 21** reaches the 4°C Hold step, transfer the first fragmented mRNA sample plate from the thermal cycler to ice or a cold block. Proceed immediately to **"Step 3. Synthesize first strand cDNA"** on page 55.

Step 3. Synthesize first strand cDNA

This step uses automation protocol **FirstStrandcDNA_XT_HS2_mRNA**.

In this step, the Bravo NGS Workstation executes preparation of the sample plate containing the mRNA 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 workstation for protocol FirstStrandcDNA_XT_HS2_RNA

1. Leave the tip boxes that were used in Cassette 1 of the Labware MiniHub during the Fragmentation protocol. Clear all other plates and tip boxes from the Labware MiniHub and BenchCel.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 19. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
5. Place red PCR plate inserts at Bravo deck positions 4 and 6.

Pre-program the thermal cycler for first strand cDNA synthesis

1. Pre-program a thermal cycler using the program in **Table 21**. Start the program, then immediately pause the program.

Table 26 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 27**. 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 mixes on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 5**.

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 27 Preparation of the First Strand cDNA master mix source plate for protocol FirstStrandcDNA_XT_HS2_RNA

Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
First Strand Master Mix (amber tube with amber cap)	Column 2 (A2-H2)	9.0 µL	13.0 µL	17.0 µL	21.0 µL	29.0 µL	53.0 µL

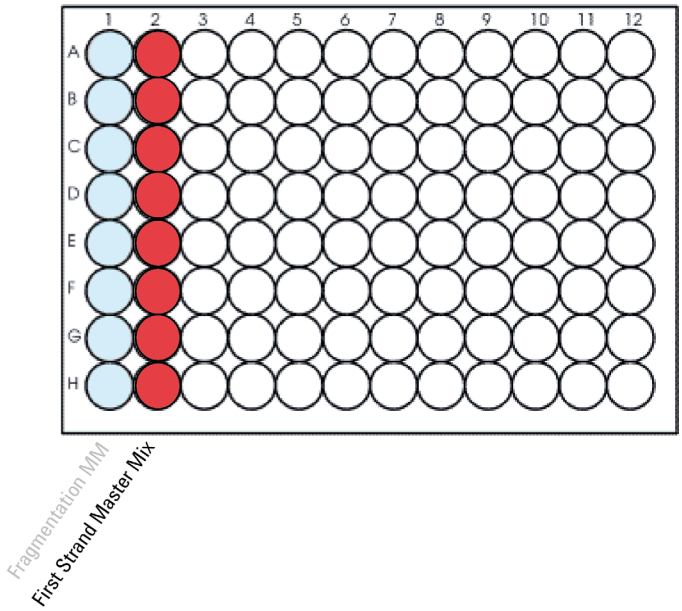


Figure 5 Configuration of the **Agilent Deep Well** source plate for protocol FirstStrandcDNA_XT_HS2_mRNA. The master mix dispensed during a previous protocol is shown in light shading.

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

3 Briefly centrifuge the plate 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 Agilent NGS Workstation

1 Load the Labware MiniHub according to **Table 18**, using the plate orientations shown in **Figure 3** on page 48.

Table 28 Initial MiniHub configuration for FirstStrandcDNA_XT_HS2_mRNA protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	New tip box from Fragmentation protocol (minus tips in column 1)	—	—	—
Shelf 1 (Bottom)	Used tip box from Fragmentation protocol (with tips in column 1) **OR** Empty tip box	—	—	Empty tip box

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

Table 29 Initial Bravo deck configuration for FirstStrandcDNA_XT_HS2_mRNA protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	Empty red insert
6	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under Parameter 2)
7	Fragmented mRNA sample plate (PCR plate type must be specified on setup form under Parameter 2)
9	First Strand cDNA master mix source plate, unsealed

3 Load the BenchCel Microplate Handling Workstation according to **Table 20**.

Table 30 Initial BenchCel configuration for FirstStrandcDNA_XT_HS2_RNA protocol

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

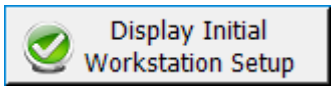
Run VWorks protocol FirstStrandcDNA_XT_HS2_mRNA

1 On the SureSelect setup form, under **Select protocol to execute**, select the **FirstStrandcDNA_XT_HS2_mRNA** protocol.

2 Under **Select labware for thermal cycling**, select the specific type of PCR plate used at positions 6 and 7 of the Bravo deck.

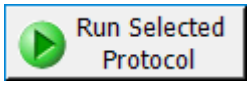
3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

4 Click **Display Initial Workstation Setup**.



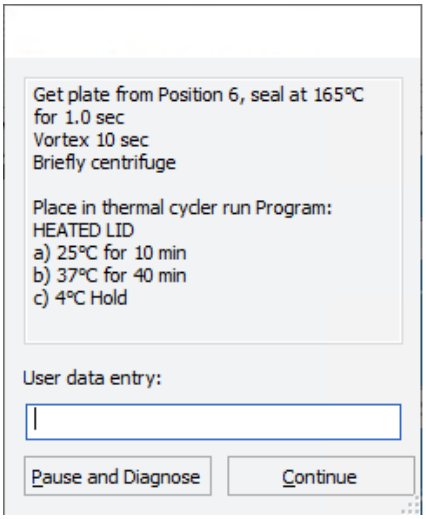
5 Verify that the NGS workstation has been set up as displayed on the right side of the form.

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



Running the FirstStrandcDNA_XT_HS2_mRNA protocol takes approximately 10 minutes.

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

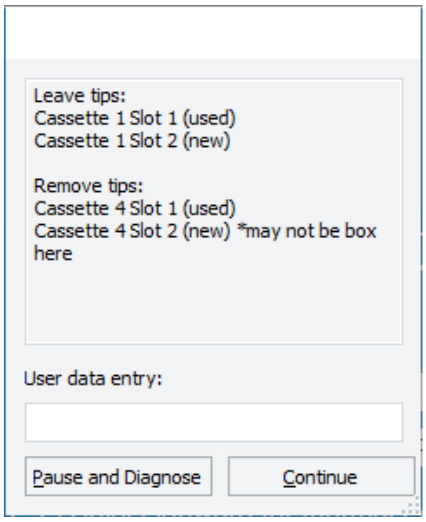


8 Briefly centrifuge the plate to drive the well contents off the walls and plate seal and to eliminate air bubbles.

9 Immediately place the sample plate in the thermal block. Close the lid and resume the thermal cycler program in **Table 26**.

10 In VWorks, click **Continue**.

The following prompt opens.



11 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 SecondStrandcDNA_XT_HS2_RNA protocol as described in **"Prepare the Second Strand master mix and master mix source plate"** on page 60.

12 On the Labware MiniHub, leave the tip boxes in Cassette 1 in place. They will be used in the SecondStrandcDNA_XT_HS2_RNA protocol as described in **"Load the Agilent NGS Workstation"** on page 62.

13 Once the thermal cycler program in **Table 26** 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 4. Synthesize and purify second strand cDNA"** on page 60.

Step 4. Synthesize and purify second strand cDNA

This step uses automation protocol **SecondStrand_XT_HS2_RNA**.

This step also uses the aliquoted plate of AMPure XP beads that was prepared on **page 36**.

In this step, the first stand cDNA is used as a template to synthesize second strand cDNA. The Agilent NGS Workstation then performs the purification steps for the cDNA using AMPure XP beads.

Prepare the workstation for runset SecondStrand_XT_HS2_RNA

1. Leave the tip boxes that were used in Cassette 1 of the Labware MiniHub during the FirstStrandcDNA_XT_HS2_mRNA protocol. Clear all other plates and tip boxes from the Labware MiniHub and BenchCel.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Pre-set the temperature of Bravo deck position 4 to 16°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
5. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
6. Place red PCR plate inserts at Bravo deck positions 4 and 9.

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 31** and using the liquid handling steps specified below.
   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 31 Preparation of Second Strand master mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Second Strand Enzyme Mix (bottle)	25 µL	265.6 µL	478.1 µL	690.6 µL	903.1 µL	1353.0 µL	2709.4 µL
Second Strand Oligo Mix (tube with yellow cap)	5 µL	53.1 µL	95.6 µL	138.1µL	180.6 µL	270.6 µL	541.9 µL
Total Volume	**30 µL**	**318.8 µL**	**573.8µL**	**828.8 µL**	**1083.8 µL**	**1623.6 µL**	**3251.3 µL**

2 Prepare the **Agilent Deep Well** source plate for the run as indicated in **Table 32**. 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 32 Preparation of the Second Strand master mix source plate for runset SecondStrandcDNA_XT_HS2_RNA

Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Second Strand Master Mix	Column 3 (A3-H3)	36.0 µL	67.0 µL	98.0 µL	129.0 µL	196.0 µL	400.0 µL

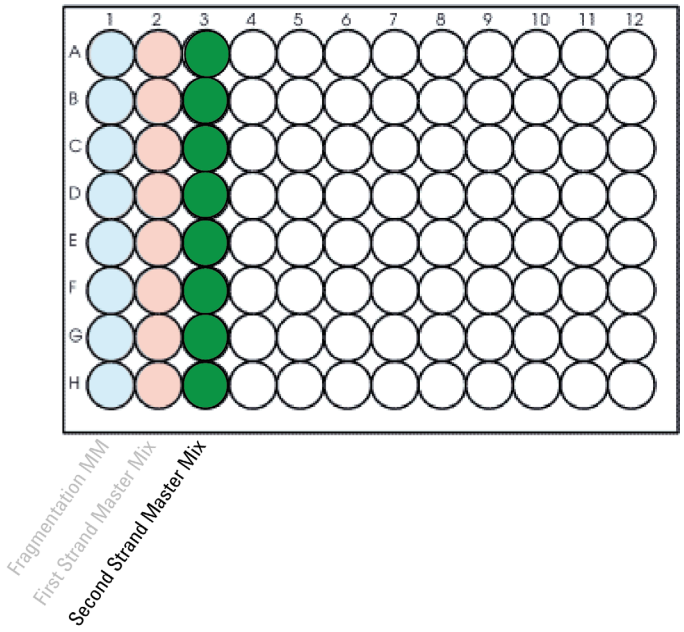


Figure 6 Configuration of the **Agilent Deep Well** source plate for runset SecondStrand_XT_HS2_RNA. The master mixes dispensed during previous protocols are shown in light shading.

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

4 Briefly centrifuge the plate 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.

Prepare the second strand synthesis reagents

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

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

At the end of the automation runset, retain this reservoir for use in the Library Preparation runset.

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

Load the Agilent NGS Workstation

1 Load the Labware MiniHub according to **Table 33**, using the plate orientations shown in **Figure 3** on page 48.

Table 33 Initial MiniHub configuration for SecondStrandcDNA_XT_HS2_RNA runset

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	Aliquoted AMPure XP beads in Agilent deep well plate from **page 36** (105 µL of beads/well)	—	—
Shelf 4	—	—	—	—
Shelf 3	—	Empty processing plate (Eppendorf twin.tec or Armadillo plate)	—	—
Shelf 2	New tip box from First Stand protocol (minus tips in columns 1 and 2)	Nuclease-free water reservoir from **step 1**	—	—
Shelf 1 (Bottom)	Used tip box from First Strand protocol (with tips in columns 1 and 2) **OR** Empty tip box	70% ethanol reservoir from **step 2**	—	Empty tip box

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

Table 34 Initial Bravo deck configuration for SecondStrand_XT_HS2_RNA runset

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	Empty red insert
5	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
6	Second Strand master mix source plate, unsealed
9	First strand cDNA samples in PCR plate seated in red insert (PCR plate type must be specified on setup form under Parameter 2)

3 Load the BenchCel Microplate Handling Workstation according to **Table 35**.

Table 35 Initial BenchCel configuration for SecondStrandcDNA_XT_HS2_RNA runset

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

Run VWorks runset SecondStrand_XT_HS2_RNA

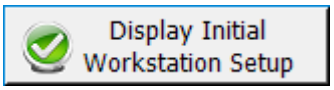
1 On the SureSelect setup form, under **Select protocol to execute**, select the **SecondStrand_XT_HS2_RNA** runset.

2 Under **Select labware for thermal cycling**, select the specific type of PCR plate used at position 9 of the Bravo deck.

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

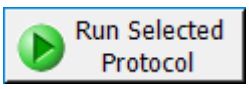
4 Verify that the **Processing Plate** selection is set to the correct plate type. See **page 22** for more information on using this segment of the form.

5 Click **Display Initial Workstation Setup**.



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

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



Running the SecondStrand_XT_HS2_RNA runset takes approximately 2 hours. Once complete, the purified cDNA samples are ready for library preparation. The samples are located in the processing plate at position 7 of the Bravo deck. Transfer the cDNA sample plate from the Bravo deck to ice or a cold block. This cDNA sample plate is later loaded back onto the Bravo deck for the Library Preparation runset (LibraryPrep_XT_HS2_ILM or LibraryPrep_LILQ_XT_HS2_ILM).

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 Library Preparation runset.

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.

6 Library Preparation

This chapter contains instructions 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 mRNA library preparation workflow, see **Figure 2** on page 26.

When using high-quality (RIN >8) total RNA samples, the protocol requires 10 ng to 1 µg of input total RNA. For total RNA samples of reduced integrity (RIN 6–8), 50 ng to 1 µg of input total RNA is required. For optimal results, use the maximum amount of input RNA available within the recommended range. Analysis using the molecular barcodes is recommended when sample is available in low amounts (10 to 50 ng).

Step 1. Prepare adaptor-ligated libraries

The step uses one of the two Library Preparation runsets offered in the SureSelect XT HS2 mRNA VWorks form: the **LibraryPrep_XT_HS2_ILM** runset or the **LibraryPrep_LILQ_XT_HS2_ILM** runset. Refer to **Table 10** on page 33 for guidance on selecting the appropriate library preparation runset based on the quality and quantity of the total RNA samples.

CAUTION When preparing the adaptor-ligated libraries, certain steps of the instructions differ depending on the Library Preparation runset you are running. Make sure to follow the instructions appropriate for your run.

This step also uses the aliquoted plate of AMPure XP beads that was prepared on **page 38**. If you are running the Library Preparation runset for low-input or low-quality samples, then an additional plate of AMPure XP beads (prepared on **page 40**) is required.

In this step, the Agilent NGS Workstation completes the DNA end modification steps required for SureSelect library preparation, including end-repair, dA-tailing, and ligation of the molecular-barcoded adaptor. After the end-modification steps, the Agilent NGS Workstation purifies the prepared DNA using AMPure XP beads.

The components listed in **Table 36** are used in this step. Thaw and mix each component as directed in **Table 36** before use. Before starting the run, you need to prepare master mixes (with overage) for each step, without the DNA sample. Master mixes for runs that include 1, 2, 3, 4, 6, and 12 columns (including overage) are shown in reagent preparation tables.

Table 36 Reagents thawed before use in the LibraryPrep_XT_HS2_ILM or LibraryPrep_LILQ_XT_HS2_ILM runset

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 workstation

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

2 Pre-set the temperature of Bravo deck position 4 to 79°C using the Inheco Multi TEC control touchscreen, as described in **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 19. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.

3 Pre-set the temperature of Bravo deck position 6 to 20°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.

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

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

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

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

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

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

Table 37 Preparation of End Repair/dA-Tailing master mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
End Repair-A Tailing Buffer (bottle)	16 µL	204 µL	340 µL	476 µL	612 µL	936 µL	1944 µL
End Repair-A Tailing Enzyme Mix (tube with orange cap)	4 µL	51 µL	85 µL	119 µL	153 µL	234 µL	486 µL
Total Volume	**20 µL**	**255 µL**	**425 µL**	**595 µL**	**765 µL**	**1170 µL**	**2430 µL**

Prepare the Ligation master mix

1 Prepare the appropriate volume of Ligation master mix, using volumes listed in **Table 38** and using the liquid handling steps specified below.

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

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

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

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

Table 38 Preparation of Ligation master mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Ligation Buffer (bottle)	23 µL	293.3 µL	488.8 µL	684.3 µL	879.8 µL	1407.6 µL	2834.9 µL
T4 DNA Ligase (tube with blue cap)	2 µL	25.5 µL	42.5 µL	59.5 µL	76.5 µL	122.4 µL	246.5 µL
Total Volume	**25 µL**	**318.8 µL**	**531.3 µL**	**743.8 µL**	**956.3 µL**	**1530 µL**	**3081.4 µL**

Prepare the Adaptor Oligo Mix

1 Prepare the appropriate volume of Adaptor Oligo Mix dilution, according to **Table 39**. Mix well using a vortex mixer and keep on ice.

Table 39 Preparation of Adaptor Oligo Mix dilution

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2.5 µL	42.5 µL	63.8 µL	85.0 µL	106.3 µL	151.4 µL	282.1 µL
XT HS2 RNA Adaptor Oligo Mix (tube with green cap)	5 µL	85.0 µL	127.5 µL	170.0 µL	212.5 µL	302.8 µL	564.2µL
Total Volume	**7.5 µL**	**127.5 µL**	**191.3 µL**	**255.0 µL**	**318.8 µL**	**454.1 µL**	**846.3 µL**

Prepare the master mix source plate

1 Prepare the **Agilent Deep Well** master mix source plate containing the mixtures prepared in **step 1** through **step 1**. Add the volumes indicated in **Table 40** 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 7**.

Table 40 Preparation of the master mix source plate for LibraryPrep_XT_HS2_ILM or LibraryPrep_LILQ_XT_HS2_ILM runset

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
End Repair-dA Tailing master mix	Column 4 (A4-H4)	31.0 µL	52.0 µL	73.0 µL	94.0 µL	140.0 µL	280.0 µL
Ligation master mix	Column 5 (A5-H5)	36.0 µL	62.0 µL	88.0 µL	114.0 µL	180.0 µL	366.5 µL
Adaptor Oligo Mix dilution	Column 6 (A6-H6)	15.0 µL	22.5 µL	30.0 µL	37.5 µL	56.3µL	105.0 µL

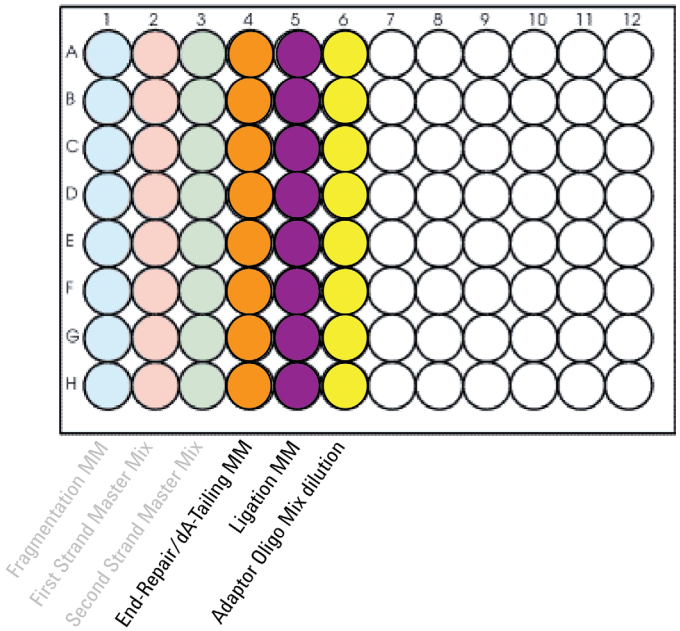


Figure 7 Configuration of the **Agilent Deep Well** master mix source plate for runset LibraryPrep_XT_HS2_ILM or LibraryPrep_LILQ_XT_HS2_ILM. The master mixes dispensed during previous protocols are shown in light shading.

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

3 Briefly centrifuge the plate to drive the well contents off the walls and plate seal and to eliminate any bubbles. Keep the master mix source plate on ice.

NOTE

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

Prepare the purification reagents

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

   Use the same Agilent shallow well reservoir that was used in the SecondStrand_XT_HS2_RNA protocol or use a fresh Agilent shallow well reservoir.

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

   At the end of the automation runset, retain this reservoir for use in the AMPureXP_XT_HS2_ILM (PCR) protocol.
2. Prepare a reservoir containing 70% ethanol. The required volume is dependent on which runset you are running.
   - For the LibraryPrep_XT_HS2_ILM runset, prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.
   - For the LibraryPrep_LILQ_XT_HS2_ILM runset, prepare an Agilent deep well reservoir containing 100 mL of freshly-prepared 70% ethanol.

Load the Agilent NGS Workstation

1. Load the Labware MiniHub according to **Table 41**, using the plate orientations shown in **Figure 3** on page 48.

NOTE

The LibraryPrep_LILQ_XT_HS2_ILM runset for low-input or low-quality samples requires an additional plate in cassette 2, shelf 5, of the Labware MiniHub that is not required for the standard LibraryPrep_XT_HS2_ILM runset.

Table 41 Initial MiniHub configuration for LibraryPrep_XT_HS2_ILM or LibraryPrep_LILQ_XT_HS2_ILM runset

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Aliquoted AMPure XP beads in Agilent deep well plate from **page 38** (80 µL of beads/well)	**For LILQ only:** Aliquoted AMPure XP beads in Agilent deep well plate from **page 40** (60 µL of beads/well)	—	—
Shelf 4	Empty processing plate (Eppendorf twin.tec or Armadillo plate)	—	—	—
Shelf 3	Empty processing plate (Eppendorf twin.tec or Armadillo plate)	Empty processing plate (Eppendorf twin.tec or Armadillo plate)	—	—
Shelf 2	New tip box from Second Stand protocol (minus tips in columns 1–3)	Nuclease-free water reservoir from **step 1**	—	—
Shelf 1 (Bottom)	Used tip box from Second Strand protocol (with tips in columns 1–3) **OR** Empty tip box	70% ethanol reservoir from **step 2**	—	Empty tip box

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

Table 42 Initial Bravo deck configuration for LibraryPrep_XT_HS2_ILM or LibraryPrep_LILQ_XT_HS2_ILM runset

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	Empty red insert
5	Empty processing plate (Eppendorf twin.tec or Armadillo plate)
7	Processing plate containing cDNA samples
9	Library Prep master mix source plate, unsealed

3 Load the BenchCel Microplate Handling Workstation according to the Library Preparation runset you are running.

- See **Table 43** for the BenchCel configuration for runset **LibraryPrep_XT_HS2_ILM**.
- See **Table 44** for the BenchCel configuration for runset **LibraryPrep_LILQ_XT_HS2_ILM**.

Table 43 Initial BenchCel configuration for runset LibraryPrep_XT_HS2_ILM

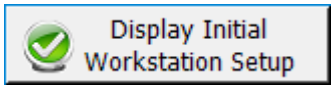
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	2 Tip boxes	—	—	—
3	2 Tip boxes	—	—	—
4	3 Tip boxes	—	—	—
6	4 Tip boxes	—	—	—
12	7 Tip boxes	—	—	—

Table 44 Initial BenchCel configuration for runset LibraryPrep_LILQ_XT_HS2_ILM

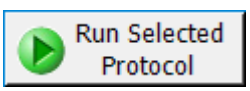
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	2 Tip boxes	—	—	—
3	3 Tip boxes	—	—	—
4	4 Tip boxes	—	—	—
6	6 Tip boxes	—	—	—
12	6 Tip boxes	5 Tip boxes	—	—

Run VWorks runset LibraryPrep_XT_HS2_ILM or LibraryPrep_LILQ_XT_HS2_ILM

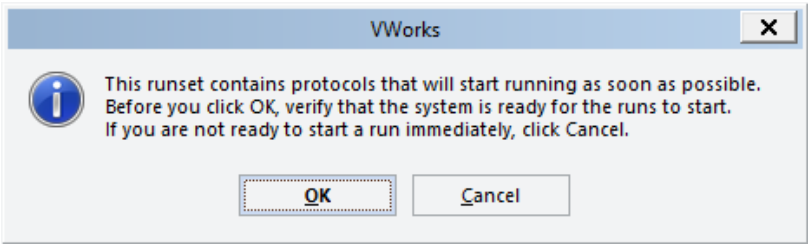
1. On the SureSelect setup form, under **Select protocol to execute**, select the appropriate runset.
   - **LibraryPrep_XT_HS2_ILM** for standard samples
   - **LibraryPrep_LILQ_XT_HS2_ILM** for low-input or low-quality samples
2. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
3. Verify that the **Processing Plate** selection is set to the correct plate type. See **page 22** for more information on using this segment of the form.
4. Click **Display Initial Workstation Setup**.



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



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



Running the Library Preparation runset takes approximately 2 hours. Once complete, the purified, adaptor-ligated cDNA samples are located in the processing plate at position 7 of the Bravo deck.

Step 2. Amplify adaptor-ligated libraries

This step uses automation protocol **PCR_XT_HS2_ILM**.

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

The components listed in **Table 45** are used in this step. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 45 Reagents for PCR amplification of the cDNA libraries

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 76**

* 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 workstation

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 19. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
5. Place a red PCR plate insert at Bravo deck position 6.

Pre-program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 46**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the automation run.

Table 46 PCR Thermal Cycler Program*

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	9 to 16 (see **Table 47** 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 47 PCR cycle number recommendations

Quantity of Input Total RNA	Cycles
1000 ng	9 cycles
250 ng	11 cycles
100 ng	12 cycles
50 ng	14 cycles
10 ng	16 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 PCR thermal cycling. Make sure to maintain the same well location for each primer pair when transferring to the PCR plate. Keep the PCR plate on ice.

The PCR plate containing the primer pairs is loaded onto the Bravo deck in **step 2** on **page 77** for the PCR_XT_HS2_ILM protocol.

Prepare the PCR master mix and master mix source plate

1 Prepare the appropriate volume of PCR master mix, according to **Table 48**. Vortex at medium speed for 15–20 seconds and keep on ice.

Table 48 Preparation of PCR Master Mix

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

2 Using a processing plate (Eppendorf twin.tec or Armadillo plate) as the master mix source plate, add the volume of PCR master mix indicated in **Table 49** to all wells of column 1. The final configuration of the master mix source plate is shown in **Figure 8**.

Table 49 Preparation of the master mix source plate (Eppendorf twin.tec or Armadillo plate) for PCR_XT_HS2_ILM protocol

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
PCR Master Mix	Column 1 (A1-H1)	22 µL	33 µL	44 µL	55 µL	77 µL	143 µL

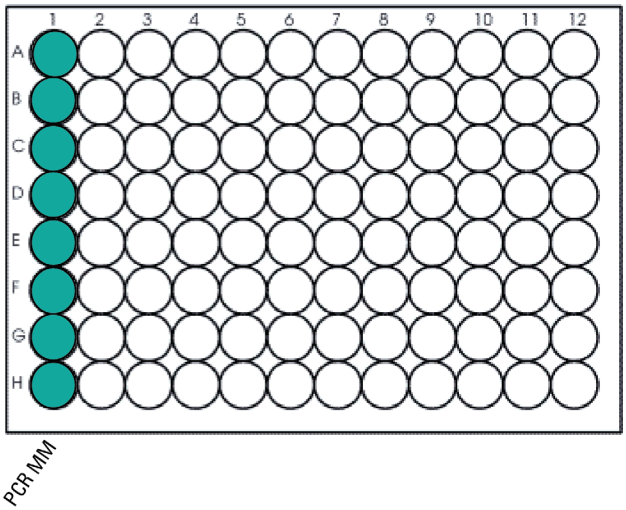


Figure 8 Configuration of the master mix source plate for protocol PCR_XT_HS2_ILM

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

4 Briefly centrifuge the plate 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 Agilent NGS Workstation

1 Load the Labware MiniHub according to **Table 50**, using the plate orientations shown in **Figure 3** on page 48.

Table 50 Initial MiniHub configuration for PCR_XT_HS2_ILM protocol

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

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

Table 51 Initial Bravo deck configuration for PCR_XT_HS2_ILM protocol

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

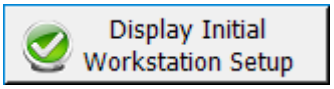
3 Load the BenchCel Microplate Handling Workstation according to **Table 52**.

Table 52 Initial BenchCel configuration for PCR_XT_HS2_ILM protocol

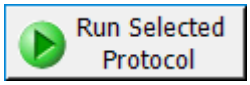
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Run VWorks protocol PCR_XT_HS2_ILM

1. On the SureSelect setup form, under **Select protocol to execute**, select the **PCR_XT_HS2_ILM** protocol.
2. Under **Select labware for thermal cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.
3. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
4. Verify that the **Processing Plate** selection is set to the correct plate type. See **page 22** for more information on using this segment of the form.
5. Click **Display Initial Workstation Setup**.

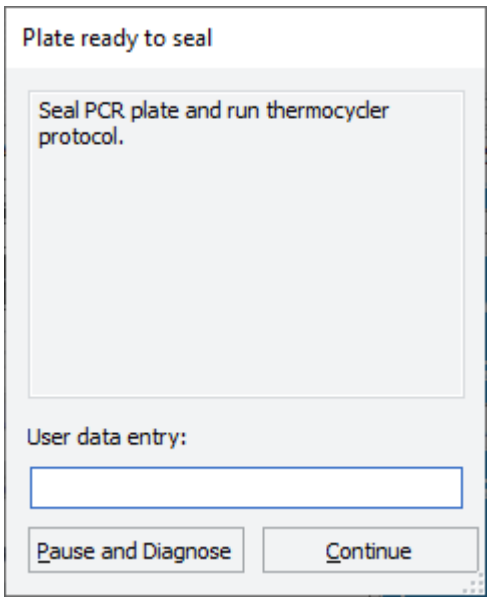


6. Verify that the NGS workstation has been set up as displayed on the right side of the form.
7. When verification is complete, click **Run Selected Protocol**.



Running the PCR_XT_HS2_ILM protocol takes approximately 15 minutes. Once complete, the PCR-ready samples, containing prepped DNA and PCR master mix, are located in the PCR plate at position 6 of the Bravo deck.

8. 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.



9. Briefly centrifuge the plate to drive the well contents off the walls and plate seal and to eliminate air bubbles.

10 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 46**. 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.

Step 3. Purify amplified cDNA using AMPure XP beads

This step uses automation protocol **AMPureXP_XT_HS2_ILM (PCR)**.

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

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

Prepare the workstation and reagents

1. Retain the master mix source plate (Eppendorf twin.tec or Armadillo plate) containing the PCR master mix located at position 9 of the Bravo deck for later use in the TS_D1000 protocol (see **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape"** on page 83). Otherwise, clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 45°C using the Inheco Multi TEC control touchscreen, as described in **"Setting the Temperature of Bravo Deck Heat Blocks"** on page 19. 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 6.
6. Prepare an Agilent shallow well reservoir containing 30 mL of 1X Low TE Buffer.

   Make sure that the buffer in the reservoir does not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.
7. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.
8. Load the Labware MiniHub according to **Table 53**, using the plate orientations shown in **Figure 3** on page 48.

Table 53 Initial MiniHub configuration for AMPureXP_XT_HS2_ILM (PCR) protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Aliquoted AMPure XP beads in Agilent Deep Well plate from **page 42** (50 µL of beads/well)	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	Empty processing plate (Eppendorf twin.tec or Armadillo plate)	—	—
Shelf 2	—	1X Low TE Buffer reservoir from **step 6**		—
Shelf 1 (Bottom)	—	70% ethanol reservoir from **step 7**	—	Empty tip box

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

Table 54 Initial Bravo deck configuration for AMPureXP_XT_HS2_ILM (PCR) protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square 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)

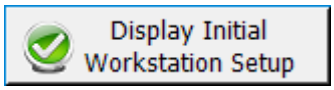
10 Load the BenchCel Microplate Handling Workstation according to **Table 55**.

Table 55 Initial BenchCel configuration for AMPureXP_XT_HS2_ILM (PCR) protocol

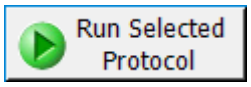
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	3 Tip boxes	—	—	—
12	5 Tip boxes	—	—	—

Run the AMPureXP_XT_HS2_ILM (PCR) protocol

1. On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_XT_HS2_ILM (PCR)** protocol.
2. Under **Select labware for thermal cycling**, select the specific type of PCR plate containing the amplified libraries at position 6.
3. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
4. Verify that the **Processing Plate** selection is set to the correct plate type. See **page 22** for more information on using this segment of the form.
5. Click **Display Initial Workstation Setup**.

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

   The purification protocol takes approximately 45 minutes. When complete, the purified cDNA samples are in the processing plate (Eppendorf twin.tec or Armadillo plate) located on Bravo deck position 7.
8. Remove the plate of purified DNA samples from deck position 7. Spin the plate briefly to collect the liquid. Keep on ice.

Step 4. 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 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 use of automation protocol TS_D1000 to prepare the D1000 assay sample plate by combining 2 µL of each DNA sample with 6 µL of D1000 sample buffer. Afterward, you transfer the prepared assay plate to the 4200 TapeStation instrument for analysis. For more information to do this step, see the Agilent D1000 Assay Quick Guide for 4200 TapeStation System.

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

Prepare the workstation and Sample Buffer source plate

1. Clear the Labware MiniHub and BenchCel of plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn off the ThermoCube device (see **page 20**) to restore position 9 of the Bravo deck to room temperature.
4. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
5. Using the same source plate that was used in the PCR_XT_HS2_ILM protocol (Eppendorf twin.tec or Armadillo plate), prepare the Sample Buffer source plate at room temperature. Add the volume of D1000 Sample Buffer indicated in **Table 56** to each well of column 2 of the plate.

Table 56 Preparation of the Sample Buffer source plate for TS_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
D1000 Sample Buffer	Column 2 (A2-H2)	11.0 µL	17.0 µL	23.0 µL	29.0 µL	41.0 µL	77.0 µL

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

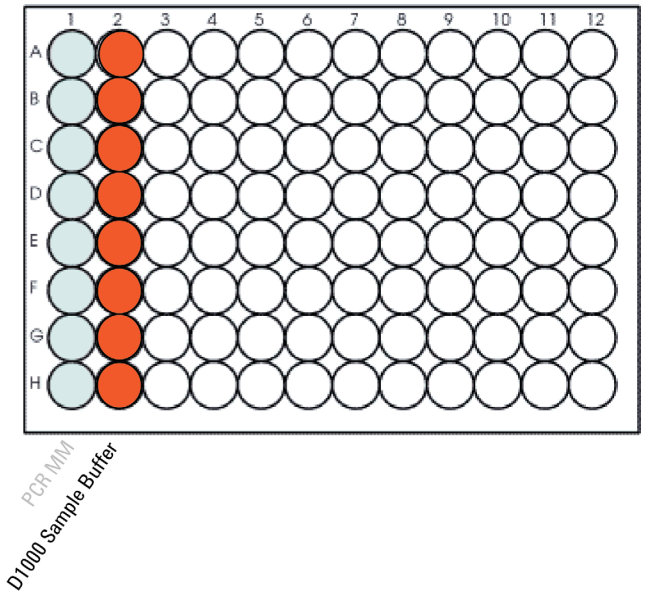


Figure 9 Configuration of the source plate (Eppendorf twin.tec or Armadillo plate) for protocol TS_D1000. The master mix dispensed during a previous protocol is shown in light shading.

Load the Agilent NGS Workstation

6 Load the Labware MiniHub according to **Table 57**, using the plate orientations shown in **Figure 3** on page 48.

Table 57 Initial MiniHub configuration for TS_D1000 protocol

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

7 Load the Bravo deck according to **Table 58**.

Table 58 Initial Bravo deck configuration for TS_D1000 protocol

Location	Content
4	Amplified libraries in processing plate (unsealed)
6	Empty TapeStation analysis plate (Agilent p/n 5042-8502)
9	Source plate containing D1000 Sample Buffer in Column 2

CAUTION

To prevent damage to the Agilent 4200 TapeStation instrument and the Agilent NGS Workstation, 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.

8 Load the BenchCel Microplate Handling Workstation according to **Table 59**.

Table 59 Initial BenchCel configuration for TS_D1000 protocol

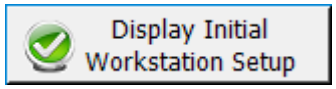
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Run VWorks protocol TS_D1000

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

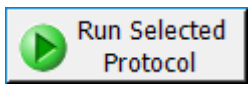
10 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

11 Click **Display Initial Workstation Setup**.



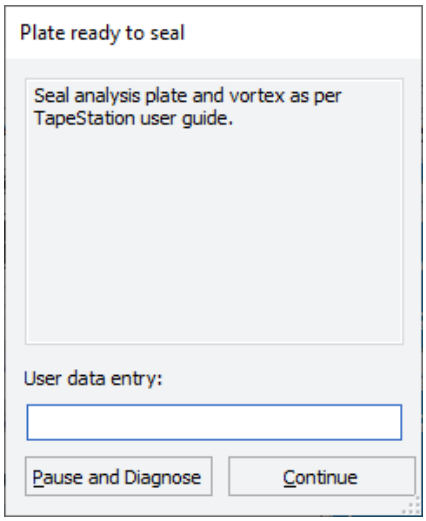
12 Verify that the NGS workstation has been set up as displayed on the right side of the form.

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



Running the TS_D1000 protocol takes approximately 15 minutes. Once removal of analytical samples is complete, remove the primary cDNA library sample plate from position 4, seal the plate, and keep on ice.

14 When prompted by VWorks as shown below, remove the Agilent 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, then vortex and spin the sealed plate as directed in the Agilent D1000 Assay Quick Guide for 4200 TapeStation System.



CAUTION

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

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

Run the D1000 Assay and analyze the data

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

16 Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 60** for guidelines). A sample electropherogram is shown in **Figure 10**.

The appearance of an additional low molecular weight peak indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to sequencing 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 117** for additional considerations.

Table 60 Library qualification guidelines

Expected library DNA fragment size peak position	NGS read lengths supported
200 to 700 bp	2 × 100 reads or 2 × 150 reads

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

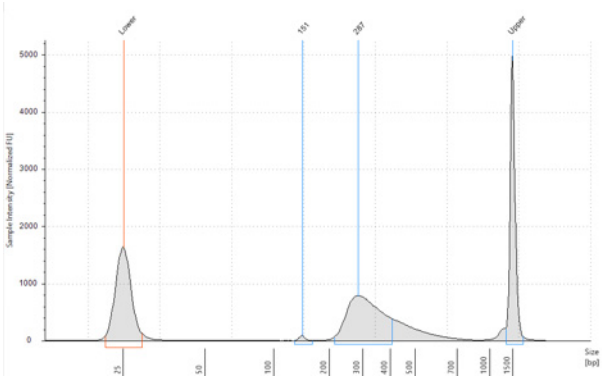


Figure 10 Final mRNA library analyzed using a D1000 ScreenTape.

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 cDNA samples on Agilent 2100 BioAnalyzer, Agilent 5200 Fragment Analyzer, or Agilent 4200/4150 TapeStation. Electropherograms obtained using one of these other analysis platforms are expected to show fragment size profiles similar to those shown for the Agilent 4200 TapeStation (see **Figure 10**). Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 60** for guidelines). **Table 61** includes links to assay instructions.

Table 61 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.

7 Guidelines for Multiplexed Sequencing

This chapter provides instructions to pool the indexed, molecular barcoded samples and provides guidelines for multiplexed sequencing.

Step 1. Pool samples for multiplexed sequencing (optional)

NOTE The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample.

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

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

Method 2 Starting with indexed libraries at different concentrations, add the appropriate volume of each library to the destination well (either manually or using the Aliquot_mRNA_Libraries 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 62 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 62 Example of volume calculation for total volume of 20 µL at 10 nM concentration

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

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

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

1 Create a .csv (comma separated value) file with the headers shown in **Figure 11**. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in .csv format. The file must include rows for all 96 wells of the plate.

2 Enter the information requested in the header for each DNA sample. **Figure 11** 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 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
14	abc	E2	A1	6.563747

Figure 11 Sample spreadsheets for method 1 and method 2

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_mRNA_ILM_v.Bx.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_mRNA_Libraries protocol run. If you are using the sample file as a template for runs with fewer than 12 columns, be sure to delete rows for any unused columns on the plate.

3 Load the .csv file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B\XT_HS2_mRNA_ILM_v.Bx.x.x\Aliquot Library Input Files**.

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

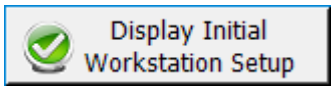
5 Load the Bravo deck according to **Table 63**.

Table 63 Initial Bravo deck configuration for Aliquot_mRNA_Libraries protocol

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)

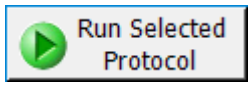
6 On the SureSelect setup form, under **Select protocol to execute**, select the **Aliquot_mRNA_Libraries** protocol.

7 Click **Display Initial Workstation Setup**.



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

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



10 When prompted, browse to the .csv file created in **step 3**, and then click **OK** to start the run.

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

11 Remove the destination plate from the Bravo deck.

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

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

Step 2. Prepare sequencing samples

The final SureSelect XT HS2 mRNA library pool is ready for direct sequencing using standard Illumina paired-end primers and chemistry. Each fragment in the prepared library contains one mRNA-derived insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform, as shown in **Figure 12**.

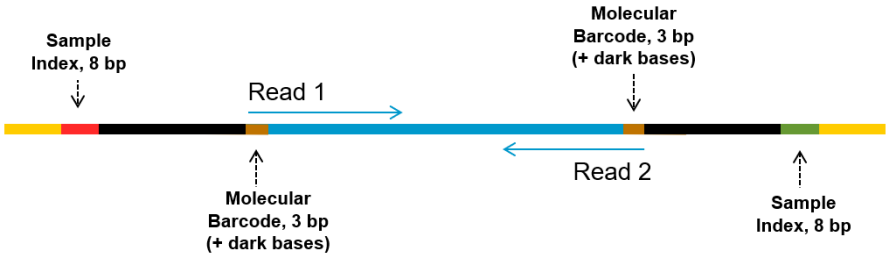


Figure 12 Content of SureSelect XT HS2 mRNA sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the dual sample indexes (red and green), dual molecular barcodes (brown) and the library bridge PCR primers (yellow).

Libraries can be sequenced on the Illumina HiSeq, MiSeq, NextSeq, or NovaSeq platform using the run type and chemistry combinations shown in **Table 64**. The optimal seeding concentration for SureSelect XT HS2 mRNA libraries varies according to sequencing platform, run type, and Illumina kit version. See **Table 64** for guidelines. Seeding concentration and cluster density may also need to be optimized based on the library DNA fragment size range and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in **Table 64**.

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

Table 64 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 3. Do the sequencing run and analyze the data

The guidelines below provide an overview of SureSelect XT HS2 RNA library sequencing run setup and analysis considerations. Links are provided for user guide sections with additional details.

- Each of the sample-level indexes requires an 8-bp index read. For complete index sequence information, see **page 102** through **page 112**.
- For the HiSeq, NextSeq, and NovaSeq platforms, set up the run using the instrument's user interface, following the guidelines on **page 94**.
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed on **page 95** to **page 97** 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 reference sequences, Illumina adaptor sequences should be trimmed from the reads using the AGeNT Trimmer module, which properly accounts for the degenerate MBCs in the adaptor sequence. See **page 98** for more information.
- Library fragments include a degenerate molecular barcode (MBC) in each strand (see **Figure 12** on page 93). 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 98** 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 98**.

HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface, using the settings shown in **Table 65**. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface and enter the Cycles settings in **Table 65**.

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 65**. In the Custom Primers section, clear (do not select) the checkboxes for all primers (*Read 1*, *Read 2*, *Index 1* and *Index 2*).

Table 65 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 72** on page 103 though **Table 79** on page 110 for nucleotide sequences of the SureSelect XT HS2 index pairs.

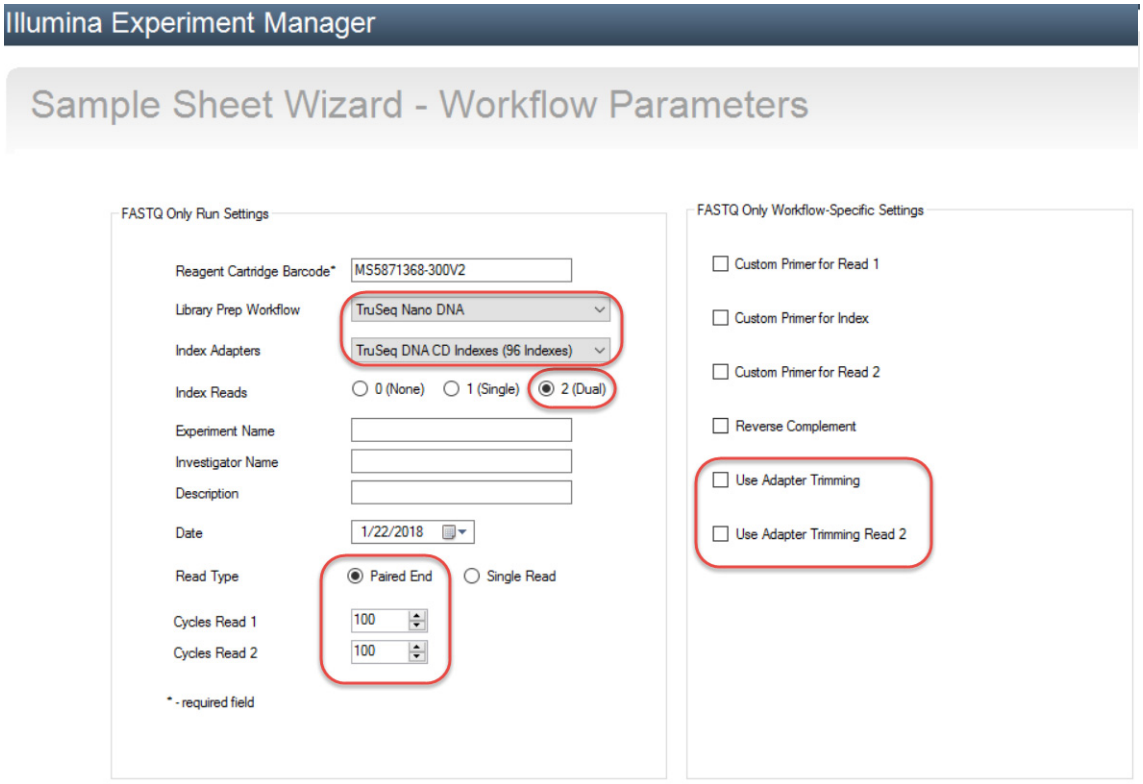
Set up a custom Sample Sheet:

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*.

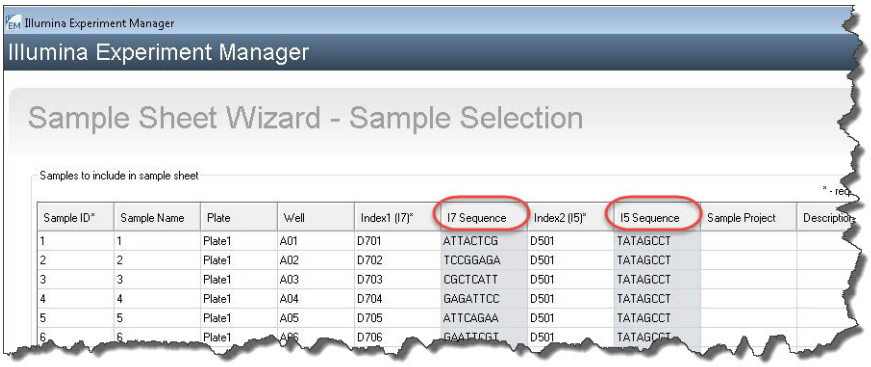
2 On the Workflow Parameters screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select TruSeq Nano DNA. In the *Index Adapters* field, select TruSeq DNA CD Indexes (96 Indexes). Make sure to clear both adaptor-trimming checkboxes under *FASTQ Only Workflow-Specific Settings* (circled below), since these are selected by default.

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 **page 103**–**page 110** 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.

[Header]								
Investigator Name	NN							
Project Name	Sequencing Project A							
Experiment Name	Experiment 1							
Date	3/20/2019							
Workflow	GenerateFASTQ							
Assay	SureSelect XT HS V2							
Chemistry	SureSelect XT HS V2							
[Reads]								
100								
100								
[Settings]								
OnlyGenerateFASTQ	1							
[Data]								
Sample_ID	Sample_Name	Sample_Plate	Sample_Well	I7_Index_ID	index	I5_Index_ID	index2	Sample_
Sample 1	Sample1	Plate1	A01	01	CAAGGTGA	01	ATGGTTAG	
Sample 2	Sample2	Plate1	A02	02	TAGACCAA	02	CAAGGTGA	
Sample 3	Sample3	Plate1	A03	03	AGTCGCGA	03	TAGACCAA	

Figure 13 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 mRNA 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 mRNA 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 12**), 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 mRNA 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

SureSelect XT HS2 mRNA Reagent Kits include the component kits listed in **Table 66**. Detailed contents of each of the multi-part component kits listed in **Table 66** are shown in **Table 67** through **Table 70** on the following pages.

Table 66 SureSelect XT HS2 mRNA Reagent Kits - Component Kits

Component Kit Name	Storage Condition	Component Kit Part Number (96 Reaction Kits)
Standard Component Modules		
SureSelect Poly-A Selection Module (Pre PCR)	+4°C	5190-6411
SureSelect cDNA Module (Pre PCR)	–20°C	5500-0149
SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0151
SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR)	–20°C	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)
Optional Component Modules		
SureSelect RNA AMPure® XP Beads	+4°C	5191-6671*

* Provided only with 96-Reaction Reagent Kit part numbers G9998A, G9998B, G9998C, G9998D.

Table 67 SureSelect Poly-A Selection Module (Pre PCR) Content

Kit Component	96 Reaction Kit Format
Oligo(dT) Microparticles	bottle
Bead Binding Buffer	bottle
Bead Washing Buffer	bottle
Bead Elution Buffer	bottle

Table 68 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 69 SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR) Content

Kit Component	96 Reaction Kit 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 70 SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) Content

Kit Component	96 Reaction Kit 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)

SureSelect XT HS2 Index Primer Pair Information

The SureSelect XT HS2 Index Primer Pairs are provided pre-combined. Each member of the primer pair contains a unique 8-bp P7 or P5 index, resulting in dual-indexed NGS libraries. One primer pair is provided in each well of 96-well plates (see **page 111** through **page 112** for plate maps). 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 72** through **Table 79**. 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 71**. 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 71 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.

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

Table 72 SureSelect XT HS2 Index Primer Pairs 1–48, provided in orange 96-well plate or in strip tubes

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 73 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 74 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 75 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 76 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 77 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 78 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 79 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

See **Table 80** on page 111 through **Table 83** on page 112 for plate maps showing positions of the SureSelect XT HS2 Index Primer Pairs.

Table 80 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 81 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 82 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 83 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 mRNA Library Preparation using Agilent NGS Workstation protocol.

Fragmentation of in-tact RNA

Table 84 Fragmentation master mix source plate for protocol Fragmentation_XT_HS2_RNA - used on page 51

Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
2X Priming Buffer (tube with purple cap)	Column 1 (A1-H1)	16.0 µL	27.0 µL	38.0 µL	49.0 µL	76.0 µL	145.0 µL

Table 85 First Strand cDNA master mix source plate for protocol FirstStrandcDNA_XT_HS2_RNA - used on page 56

Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
First Strand Master Mix (amber tube with amber cap)	Column 2 (A2-H2)	9.0 µL	13.0 µL	17.0 µL	21.0 µL	29.0 µL	53.0 µL

Table 86 Second Strand master mix - used on page 61

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Second Strand Enzyme Mix (bottle)	25 µL	265.6 µL	478.1 µL	690.6 µL	903.1 µL	1353.0 µL	2709.4 µL
Second Strand Oligo Mix (tube with yellow cap)	5 µL	53.1 µL	95.6 µL	138.1µL	180.6 µL	270.6 µL	541.9 µL
Total Volume	**30 µL**	**318.8 µL**	**573.8µL**	**828.8 µL**	**1083.8 µL**	**1623.6 µL**	**3251.3 µL**

Table 87 Second Strand master mix source plate for runset SecondStrandcDNA_XT_HS2_RNA - used on page 61

Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Second Strand Master Mix	Column 3 (A3-H3)	36.0 µL	67.0 µL	98.0 µL	129.0 µL	196.0 µL	400.0 µL

Library Preparation

Table 88 End Repair/dA-Tailing master mix - used on page page 67

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
End Repair-A Tailing Buffer (bottle)	16 µL	204 µL	340 µL	476 µL	612 µL	936 µL	1944 µL
End Repair-A Tailing Enzyme Mix (tube with orange cap)	4 µL	51 µL	85 µL	119 µL	153 µL	234 µL	486 µL
Total Volume	**20 µL**	**255 µL**	**425 µL**	**595 µL**	**765 µL**	**1170 µL**	**2430 µL**

Table 89 Ligation master mix - used on page page 68

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Ligation Buffer (bottle)	23 µL	293.3 µL	488.8 µL	684.3 µL	879.8 µL	1407.6 µL	2834.9 µL
T4 DNA Ligase (tube with blue cap)	2 µL	25.5 µL	42.5 µL	59.5 µL	76.5 µL	122.4 µL	246.5 µL
Total Volume	**25 µL**	**318.8 µL**	**531.3 µL**	**743.8 µL**	**956.3 µL**	**1530 µL**	**3081.4 µL**

Table 90 Adaptor Oligo Mix dilution - used on page page 68

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2.5 µL	42.5 µL	63.8 µL	85.0 µL	106.3 µL	151.4 µL	282.1 µL
XT HS2 RNA Adaptor Oligo Mix (tube with green cap)	5 µL	85.0 µL	127.5 µL	170.0 µL	212.5 µL	302.8 µL	564.2µL
Total Volume	**7.5 µL**	**127.5 µL**	**191.3 µL**	**255.0 µL**	**318.8 µL**	**454.1 µL**	**846.3 µL**

Table 91 Master mix source plate for LibraryPrep_XT_HS2_ILM or LibraryPrep_LILQ_XT_HS2_ILM runset - used on page page 69

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Agilent Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
End Repair-dA Tailing master mix	Column 4 (A4-H4)	31.0 µL	52.0 µL	73.0 µL	94.0 µL	140.0 µL	280.0 µL
Ligation master mix	Column 5 (A5-H5)	36.0 µL	62.0 µL	88.0 µL	114.0 µL	180.0 µL	366.5 µL
Adaptor Oligo Mix dilution	Column 6 (A6-H6)	15.0 µL	22.5 µL	30.0 µL	37.5 µL	56.3µL	105.0 µL

PCR

Table 92 PCR Master Mix - used on page page 76

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

Table 93 Master mix source plate (Eppendorf twin.tec or Armadillo plate) for PCR_XT_HS2_ILM protocol - used on page page 76

Master Mix Solution	Position on Source Plate	Volume of master mix added per Well of Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
PCR Master Mix	Column 1 (A1-H1)	22 µL	33 µL	44 µL	55 µL	77 µL	143 µL

Table 94 Sample Buffer source plate for TS_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
D1000 Sample Buffer	Column 2 (A2-H2)	11.0 µL	17.0 µL	23.0 µL	29.0 µL	41.0 µL	77.0 µL

Quick Reference Tables for Other Reagent Volumes

This section contains tables the summarize the RNA 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 95 RNA input quantity and recommended Library Preparation runset

RIN of RNA sample	RNA input quantity
>8	100 ng to 1 µg
	10 ng to 100 ng
6–8	50 ng to 1 µg
<6	Not recommended for use

Table 96 XT HS2 Index Primer Pairs Volume on Primer Plate

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

Table 97 AMPure XP Bead Volumes for AMPure XP Protocols

Protocol or Runset	Volume of AMPure Beads per Well*
SecondStrand_XT_RNA	105 µL
LibraryPrep_XT_HS2_ILM	80 µL
LibraryPrep_LILQ_XT_HS2_ILM	80 µL and 60 µL
AMPureXP_XT_HS2_ILM (PCR)	50 µL

* When preparing the plates of AMPure XP beads, fill the columns of the reservoir with enough of the bead suspension to cover the pyramid-shaped wells

Table 98 Water and Ethanol Volumes for AMPure XP Protocols

Reagent	Volume per Reservoir
70% ethanol in Agilent deep well reservoir	50 mL (or 100 mL for the LibraryPrep_LILQ_XT_HS2_ILM runset)
Nuclease-free water in Agilent shallow well reservoir	30 mL

Troubleshooting Guide

If yield of 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 PCR cycle number by 1 to 2 cycles.
- ✔ 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.
- ✔ Repeat library DNA concentration determination using a high-sensitivity assay. Visit the Automated Electrophoresis pages at agilent.com for information on the high-sensitivity DNA analysis kits available for your platform.

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 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 AMPure XP bead plates, 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 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 with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to the samples analyzed on **page 87**. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield.

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In This Book

This guide contains instructions for using the SureSelect XT HS2 mRNA Reagent Kits to prepare NGS libraries for the Illumina platform using automation protocols provided with the Agilent NGS Workstation Option B.

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