# SureSelect XT HS2 RNA System

## Automated using Agilent NGS Workstation Option B

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

# Protocol

**Version A1, September 2022**

SureSelect platform manufactured with Agilent SurePrint technology.

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

# Notices



## Manual Part Number

G9993-90010

## Edition

Version A1, September 2022

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## Acknowledgment



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# In this Guide...

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

This chapter contains an orientation to the Agilent NGS Workstation, an overview of workflow, and considerations for designing experiments for automated processing.

## 3 Preparation of AMPure XP Bead Plates

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

## 4 Preparation of Input RNA and Conversion to cDNA

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

## 5 Library Preparation

This chapter describes the steps to prepare indexed, molecular-barcoded gDNA sequencing libraries for target enrichment.

## 6 Hybridization

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

## 7 Post-Capture Sample Processing for Multiplexed Sequencing

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

## 8 Reference

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

## What's New in Version A1

- Design ID information added to **Table 4** on page 14 for pre-designed SureSelect probes.
- Updated **"Notice to Purchaser."**
- Updates to SureSelect XT HS2 Index Primer Pair information on **page 150** through **page 158** to clarify P5 index sequence orientation usage.
- Updates to downstream sequencing support information (see **page 140** through **page 144**). Key updates include support for the new CReaK tool, replacing the LocatIt tool in AGeNT v3.0 (see **page 144**), and instructions for MBC trimming using BCL Convert software (see *Note* on **page 144**).

# Content

# 1 Before You Begin



Make sure you have the most current protocol. Go to **www.agilent.com** and search for G9993-90010.

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

**NOTE** This protocol describes automated RNA sample processing for SureSelect XT HS2 Target Enrichment using the Agilent NGS Workstation Option B. For non-automated sample processing procedures see publication G9989-90000 (for post-capture pooling workflow) or G9993-90000 (for pre-capture pooling workflow).

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

# Procedural Notes

- Certain protocol steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the Agilent NGS 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 4** on page 50.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Use best-practices to prevent PCR product contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR work areas and use dedicated supplies and reagents in each area. In particular, never use materials designated to post-PCR work for pre-PCR segments of the workflow. Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
  2. Maintain clean work areas. Clean pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution.
  3. Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.
- In general, follow Biosafety Level 1 (BSL1) safety rules.
- Possible stopping points, where samples may be stored at –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.

# Safety Notes

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

# Materials Required

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

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

**Table 1 Required Reagents--All Workflow Types**

| Description | Vendor and part number |
|---|---|
| AMPure XP Kit*<br>60 mL<br>450 mL | Beckman Coulter Genomics<br>p/n A63881<br>p/n A63882 |
| Dynabeads MyOne Streptavidin T1*<br>10 mL<br>50 mL | Thermo Fisher Scientific<br>p/n 65602<br>p/n 65604D |
| 1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA) | Thermo Fisher Scientific p/n 12090-015, or equivalent |
| 100% Ethanol (Ethyl Alcohol, 200 proof) | Millipore p/n EX0276 |
| Nuclease-free Water (not DEPC-treated) | Thermo Fisher Scientific p/n AM9930 |
| QPCR Human Reference Total RNA<br>–for use as control input RNA (optional) | Agilent p/n 750500 |
| Tween 20<br>–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 RNA Reagent Kits that include SureSelect RNA AMPure® XP Beads and SureSelect Streptavidin Beads (Agilent p/n G9992A, G9992B, G9992C, or G9992D).

**Table 2 Required Equipment--All Sample and Workflow Types**

| Description | Vendor and Part Number |
|---|---|
| Agilent NGS Workstation Option B<br><br>Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com | Agilent p/n G5522A (VWorks software version 13.1.0.1366<br>OR<br>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<br>*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:<br>• 96 ABI PCR half-skirted plates (MicroAmp Optical plates), Thermo Fisher Scientific p/n N8010560<br>• 96 Agilent semi-skirted PCR plate, Agilent p/n 401334<br>• 96 Eppendorf Twin.tec half-skirted PCR plates, Eppendorf p/n 951020303<br>• 96 Eppendorf Twin.tec PCR plates (full-skirted), Eppendorf p/n 951020401<br>• 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 2 Required Equipment--All Sample and Workflow Types (continued)**

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

**Table 3 Agilent SureSelect XT HS2 RNA Reagent Kits**

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

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

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

**Table 4 Compatible Probes based on Pooling Method**

| Probe Capture Library | | Design Target | Design ID | Ordering Information |
|---|---|---|---|---|
| **Custom Probes*** | | | | |
| ***Pre-Capture* Pooling** | SSEL PreCap Custom Tier1 1–499 kb (6 Hybs† or 30 Hybs‡) | Please visit the SureDesign website to design Custom SureSelect RNA probes and obtain ordering information. Contact the SureSelect support team (see **page 2**) or your local representative if you need assistance. | | |
| | SSEL PreCap Custom Tier2 0.5 –2.9 Mb (6 Hybs† or 30 Hybs‡) | | | |
| | SSEL PreCap Custom Tier3 3 –5.9 Mb (6 Hybs† or 30 Hybs‡) | | | |
| | SSEL PreCap Custom Tier4 6 –11.9 Mb (6 Hybs† or 30 Hybs‡) | | | |
| | SSEL PreCap Custom Tier5 12–24 Mb (6 Hybs† or 30 Hybs‡) | | | |
| ***Post-Capture* Pooling** | SureSelect Custom Tier1 1–499 kb | | | |
| | SureSelect Custom Tier2 0.5–2.9 Mb | | | |
| | SureSelect Custom Tier3 3–5.9 Mb | | | |
| | SureSelect Custom Tier4 6–11.9 Mb | | | |
| | SureSelect Custom Tier5 12–24 Mb | | | |
| **Pre-designed Probes** | | | | |
| ***Pre-Capture* Pooling** | SureSelect XT HS PreCap Human All Exon V8 (12 Hybs)** | Genome | S33266340 | Agilent p/n 5191-6878 |
| | SureSelect XT HS Pre-Cap Human All Exon V8 +UTR (12 Hybs)** | Genome | S33613271 | Agilent p/n 5191-7406 |
| | SureSelect Pre-Capture Pooling Human All Exon V7 (12 Hybs)** | Genome | S31285117 | Agilent p/n 5191-5735 |
| | SureSelect XT2 Clinical Research Exome V2 (12 Hybs)** | Genome | S30409818 | Agilent p/n 5190-9501 |
| | SureSelect XT2 Mouse All Exon (12 Hybs)** | Genome | S0276129 | Agilent p/n 5190-4682 |
| | ClearSeq Inherited Disease XT2 (12 Hybs)** | Genome | S0684402 | Agilent p/n 5190-7525 |
| | ClearSeq Comprehensive Cancer XT2 (6 Hybs)† | Genome | 0425761 | Agilent p/n 5190-8018 |
| ***Post-Capture* Pooling** | SureSelect XT HS Human All Exon V8, 96 Reactions | Genome | S33266340 | Agilent p/n 5191-6875 |
| | SureSelect XT HS Human All Exon V8+UTR, 96 Reactions | Genome | S33613271 | Agilent p/n 5191-7403 |
| | SSel XT HS and XT Low Input Human All Exon V7, 96 Reactions | Genome | S31285117 | Agilent p/n 5191-4029 |
| | SureSelect XT Clinical Research Exome V2, 96 Reactions | Genome | S30409818 | Agilent p/n 5190-9492 |
| | SureSelect XT Mouse All Exon, 96 Reactions | Genome | S0276129 | Agilent p/n 5190-4642 |
| | ClearSeq Comprehensive Cancer XT, 96 Reactions | Genome | 0425761 | Agilent p/n 5190-8012 |
| | ClearSeq Inherited Disease XT, 96 Reactions | Genome | S0684402 | Agilent p/n 5190-7519 |
| | ClearSeq RNA Kinome, 96 Reactions | Transcriptome | 0320691 | Agilent p/n 5190-4802 |

**Table 4 Compatible Probes based on Pooling Method (continued)**

| Probe Capture Library | | Design Target | Design ID | Ordering Information |
|---|---|---|---|---|
| **Pre-designed Probes customized with additional *Plus* custom content** | | | | |
| ***Pre-Capture* Pooling** | SureSelect XT2 Clinical Research Exome V2 Plus 1 (12 Hybs)** | Genome | | |
| | SureSelect XT2 Clinical Research Exome V2 Plus 2 (12 Hybs)** | Genome | | |
| | ClearSeq Comprehensive Cancer Plus XT2 (6 Hybs)† | Genome | | |
| | ClearSeq Inherited Disease Plus XT2 (12 Hybs)** | Genome | Please visit the SureDesign website to design the customized *Plus* content and obtain ordering information. Contact the SureSelect support team (see **page 2**) or your local representative if you need assistance. | |
| ***Post-Capture* Pooling** | SSel XT HS and XT Low Input Human All Exon V7 Plus 1 | Genome | | |
| | SSel XT HS and XT Low Input Human All Exon V7 Plus 2 | Genome | | |
| | SureSelect XT Clinical Research Exome V2 Plus 1 | Genome | | |
| | SureSelect XT Clinical Research Exome V2 Plus 2 | Genome | | |
| | ClearSeq Comprehensive Cancer Plus XT | Genome | | |
| | ClearSeq Inherited Disease Plus XT | Genome | | |

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

† The 6-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 16 samples/pool (for 6 hybs × 16 samples/hyb). Contains enough reagent for 6 hybridization reactions using the run setup on **page 105**. The hybridization protocol can only process columns in which all 8 wells contain a hybridization reaction. In order to have enough reagent to run 3 full columns (i.e., 24 wells), you must purchase 4 units of the 6-Hyb Probe.

‡ The 30-Hyb Probes support target enrichment for 480 samples pooled using the configuration recommended for the included designs of 16 samples/pool (for 30 hybs × 16 samples/hyb). Contains enough reagent for 30 hybridization reactions using the run setup on **page 105**. The hybridization protocol can only process columns in which all 8 wells contain a hybridization reaction.

** The 12-Hyb Probes support target enrichment for 96 samples pooled using the configuration recommended for the included designs of 8 samples/pool (for 12 hybs × 8 samples/hyb). Contains enough reagent for 6 hybridization reactions using the run setup on **page 105**. The hybridization protocol can only process columns in which all 8 wells contain a hybridization reaction. In order to have enough reagent to run 3 full columns (i.e., 24 wells), you must purchase 2 units of the 12-Hyb Probe.

**Table 5 Nucleic Acid Analysis Platform Options--Select One**

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

# 2 Using the Agilent NGS Workstation for SureSelect Target Enrichment



This chapter contains an orientation to the Agilent NGS Workstation, an overview of the SureSelect XT HS2 target enrichment 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 6**.

Review the user guides listed in **Table 6** (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 Target Enrichment workflow are detailed in this user guide.

**Table 6 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.

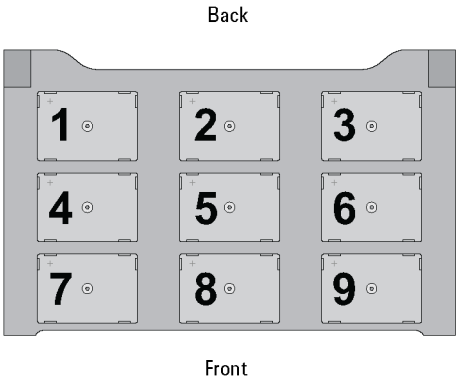



**Figure 1** Bravo platform deck

### Setting the Temperature of Bravo Deck Heat Blocks

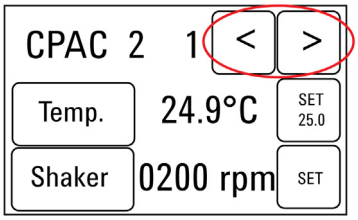
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the 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 7** for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

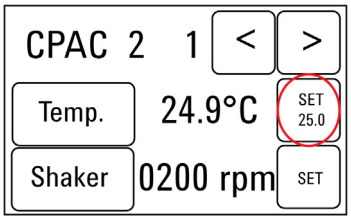
**Table 7 Inheco Multi TEC Control touchscreen designations**

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

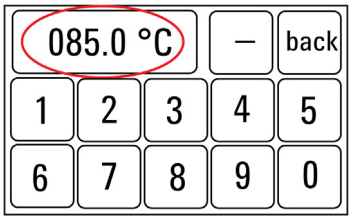
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



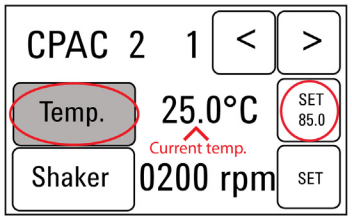

2 To set the temperature of the selected block, press the SET button.



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




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




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

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

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

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

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

3 Press the START button.

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

## VWorks Automation Control Software

VWorks software, included with your Agilent NGS 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 RNA 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.

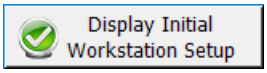
### Using the SureSelect XT HS2 RNA Form to setup and start a run

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

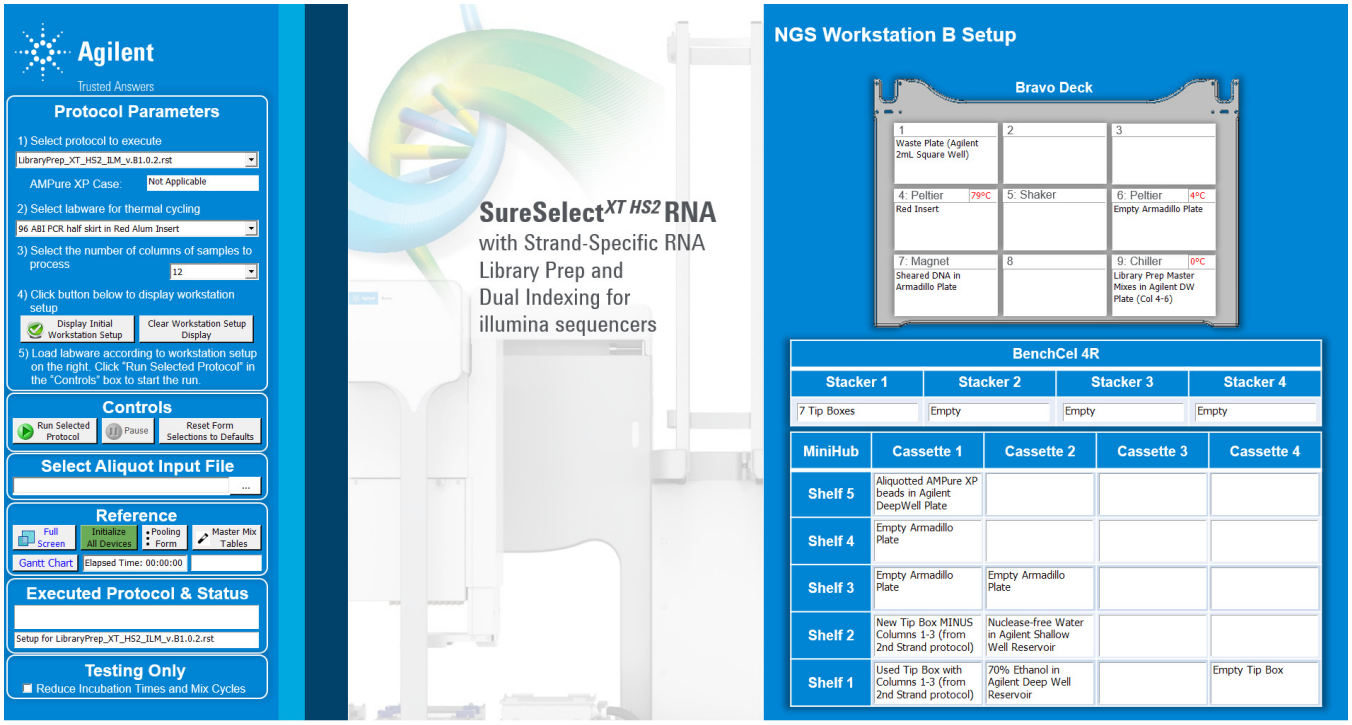

1 Open the form using the shortcut (shown below) on your desktop, or by opening the file **XT_HS2_RNA_ILM_v.Bx.x.x.VWForm** in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_RNA_ILM_v.Bx.x.x\Forms** (where x.x.x is the version number).


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

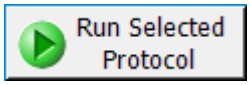
3 Once all run parameters have been specified on the form, click **Display Initial Workstation Setup**.




4 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.



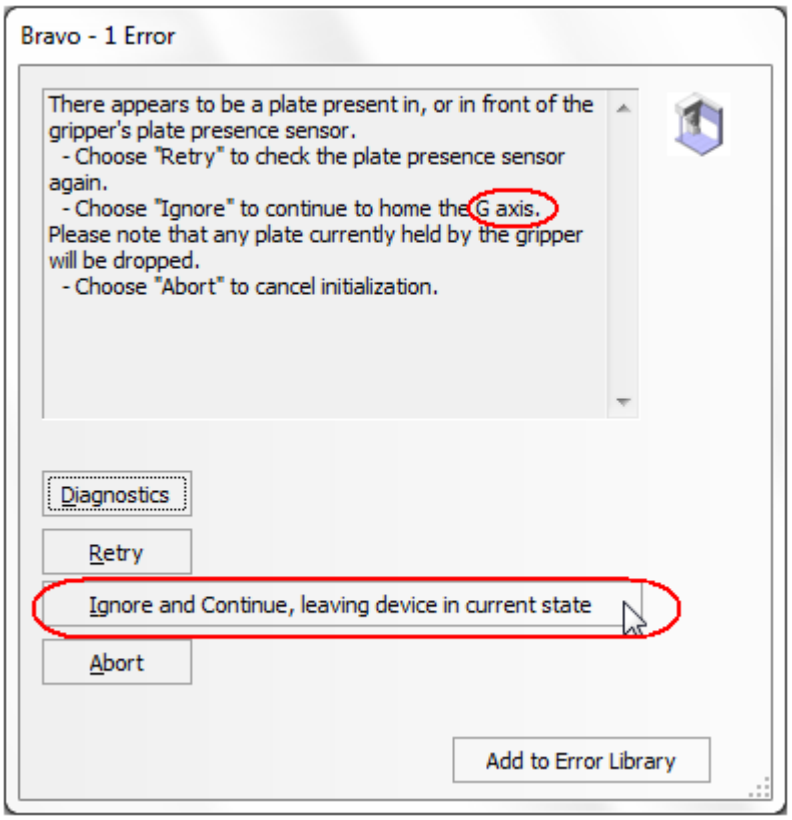

5 After verifying that the NGS Workstation has been set up correctly, click **Run Selected Protocol**.



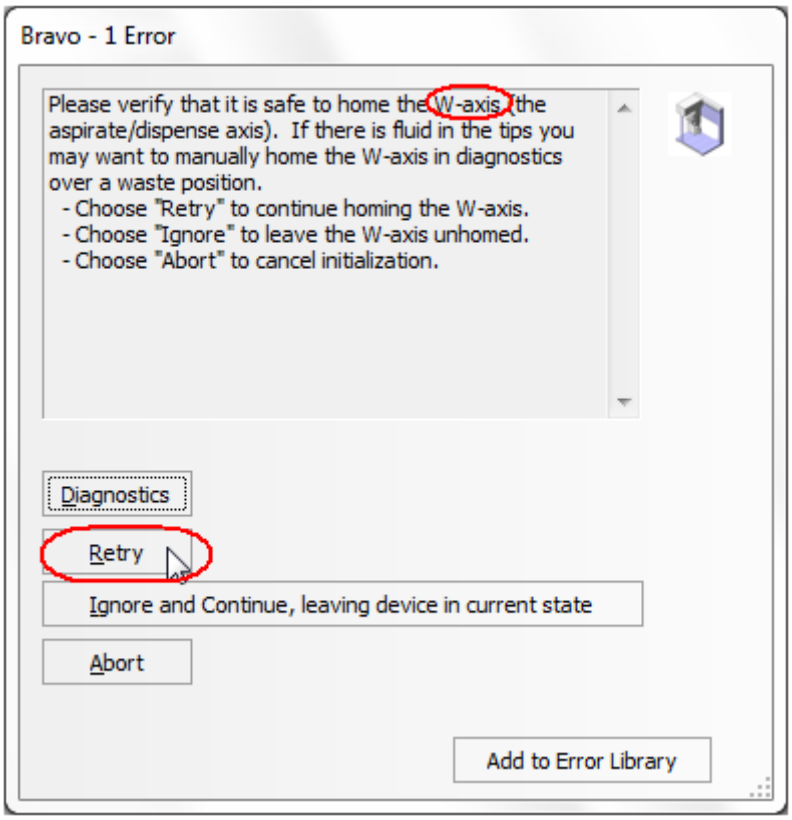

### Error messages encountered at start of run

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.



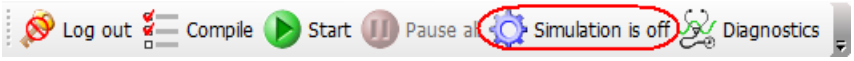
2 If you encounter the W-axis error message shown below, select **Retry**.




### 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_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

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

Agilent offers four different plates of index pairs for use with the SureSelect XT HS2 RNA DNA library preparation reagents to allow for multiplexed sequencing (refer to **"Index Primer Pair Plate Maps"** on page 159). Depending on the SureSelect XT HS2 RNA Reagent Kit(s) used in the protocol, you can pool samples for multiplexed sequencing either prior to hybridization with the Probe (i.e., pre-capture pooling) or after hybridization and subsequent PCR amplification of the captured libraries (i.e., post-capture pooling).

See **Table 9** for a summary of the VWorks protocols used during the workflow. Then, see **Preparation of AMPure XP Bead Plates**, **Library Preparation**, **Hybridization**, and **Post-Capture Sample Processing for Multiplexed Sequencing** chapters for complete instructions for use of the VWorks protocols for sample processing.

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

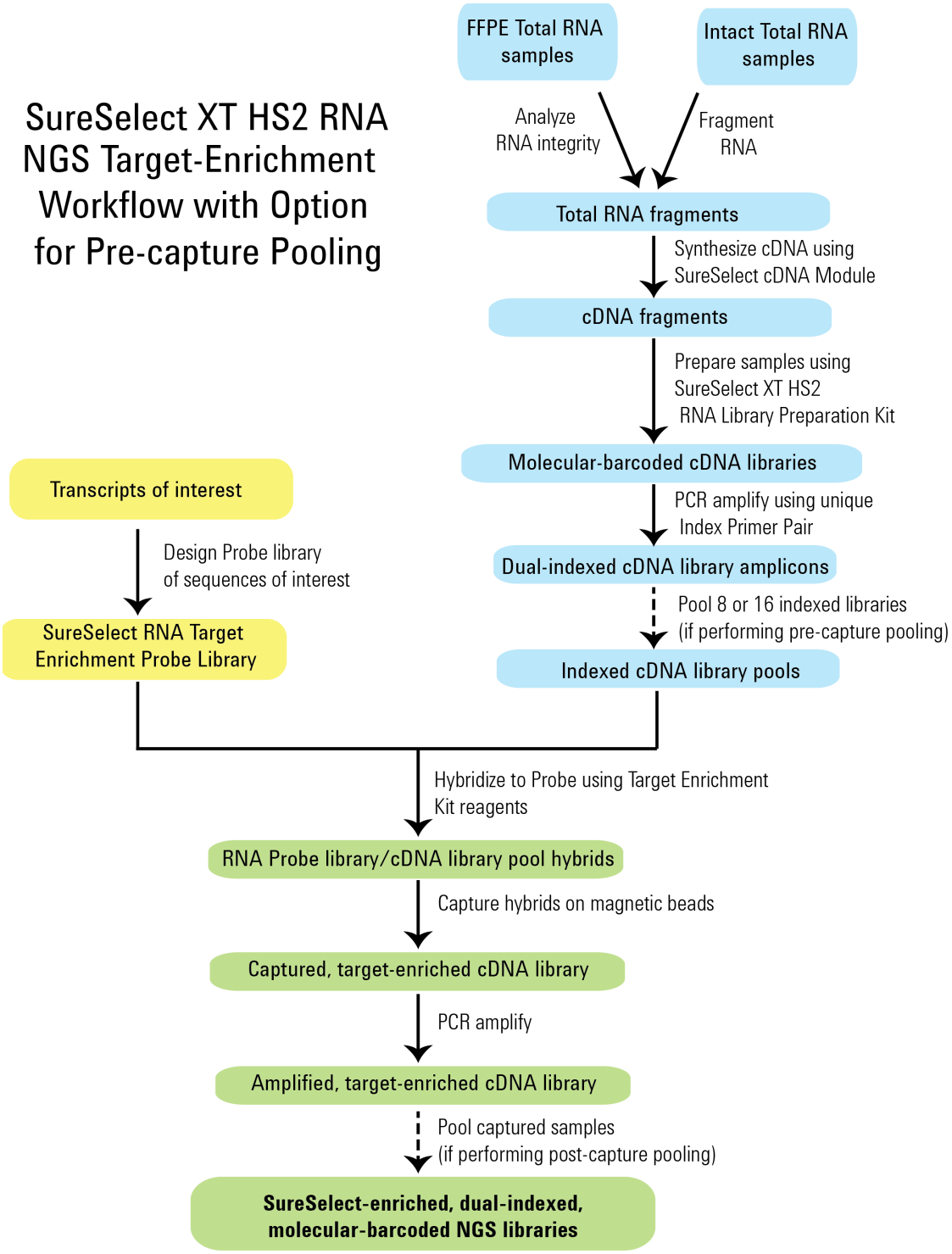



**Figure 2** Overall sequencing sample preparation workflow

## Workflow Modulations

The SureSelect XT HS2 RNA target enrichment workflow can be modulated for different applications as described below and summarized in **Table 8** on page 28.

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

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

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

**Table 8 Summary of workflow modulations supported by the automation protocols**

| Property | Options | Usage Notes |
|---|---|---|
| RNA Sample Integrity | Intact RNA | After the automated fragmentation protocol, transfer the plate of RNA samples to a thermal cycler for heat fragmentation at 94°C as described in **Table 20** on page 48. |
| | FFPE RNA | Qualify RNA before use in assay; see **"Assess initial quality of RNA samples (FFPE RNA only)"** on page 46. After the automated fragmentation protocol, do not transfer the plate to a thermal cycler for heat fragmentation. |
| Pooling Strategy | Pre-Capture Pooling | For library preparation, use a SureSelect XT HS2 RNA Library Preparation Kit that is compatible with pre-capture pooling (e.g., Agilent part numbers G9993A through G9993D). For target enrichment, use the SureSelect XT HS2 RNA Target Enrichment Kit. Refer to **Table 3** on page 13. |
| | Post-Capture Pooling | Use one of the SureSelect XT HS2 RNA Reagent Kits compatible with post-capture pooling (Agilent part numbers G9991A through G9991D, or G9992A through G9992D). Kits include reagents for both library preparation and target enrichment. Refer to **Table 3** on page 13. |

# Automation Protocols used in the Workflow

**Table 9 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 Pre-Capture PCR purification protocol | AMPureXP_Aliquot (Case Pre-Capture PCR) |
| | Aliquot AMPure XP Beads for use in the Pre-Capture Pooling protocol for concentrating the DNA | AMPureXP_Aliquot (Case Concentration of Pool) |
| | Aliquot AMPure XP Beads for use in the Post-Capture PCR purification protocol | AMPureXP_Aliquot (Case Post-Capture PRR) |
| RNA Preparation and cDNA Conversion | Mix RNA samples with the 2X Priming Buffer | Fragmentation_XT_HS2_RNA |
| | Synthesize first-strand cDNA | FirstStrandcDNA_XT_HS2_RNA |
| | Synthesize and purify second-strand cDNA | SecondStrand_XT_HS2_RNA |
| Library Preparation | Prepare duplex, molecular-barcoded DNA libraries | Runset LibraryPrep_XT_HS2_ILM |
| | Amplify indexed DNA libraries with unique dual indexing primer pair | Pre-CapPCR_XT_HS2_ILM |
| | Purify indexed DNA libraries using AMPure XP beads using an elution volume suitable for single-plexed hybridization (i.e., the post-capture pooling workflow) | AMPureXP_XT_HS2_ILM (Case Pre-Capture PCR – SinglePlex) |
| | Purify indexed DNA libraries using AMPure XP beads using an elution volume suitable for multi-plexed hybridization (i.e., the pre-capture pooling workflow) | AMPureXP_XT_HS2_ILM (Case Pre-Capture PCR – MultiPlex) |
| | Analyze indexed DNA libraries using Agilent TapeStation platform | TS_D1000 |
| Library Pooling (for **pre-capture pooling** workflow) | Pool indexed DNA libraries in pools of 8 or 16 | PreCapture_Pooling<br>*This protocol is set up and executed from the XT HS2 Pooling VWorks Form* |
| Multi-Plex Pre-Hybridization (for **pre-capture pooling** workflow) | Dilute pooled samples of indexed DNA libraries to normalize volumes to 100 µL | Aliquot_Water |
| | Concentrate pooled samples to 24 µL for hybridization | AMPureXP_XT_HS2_ILM (Case Concentration of Pool) |
| Single-Plex Pre-Hybridization (for **post-capture pooling** workflow) | Aliquot 200 ng of prepped libraries | Aliquot_Libraries |
| Hybridization and Capture | Hybridize prepped libraries or library pools (target enrichment) | Hyb_XT_HS2_ILM |
| | Capture and wash DNA hybrids | Runset SSELCapture&Wash_XT_HS2 |

**Table 9 Overview of VWorks protocols and runsets (continued)**

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

# Experimental Setup Considerations for Automated Runs

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

**Table 10 Columns to Samples Equivalency**

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

The number of columns or samples that may be processed using the supplied reagents (see **Table 1**) 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

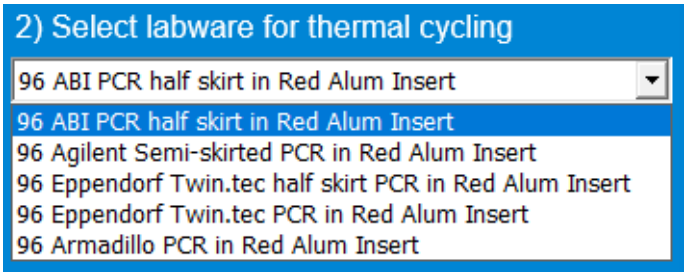
- The Agilent NGS Workstation processes samples column-wise beginning at column 1. The samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- At the hybridization step (see **Figure 2**), you can add a different Probe to each row of the plate. Plan your experiment such that each prepared DNA library corresponds to the appropriate probe row in the sample plate.
- For post-capture amplification (see **Figure 2**), different probes can require different amplification cycle numbers, based on the probe design sizes. It is most efficient to process similar-sized probes on the same plate. See **Table 86** on page 121 to determine which probes may be amplified on the same plate.

## Considerations for Equipment Setup

- Some workflow steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the 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 11**.




**CAUTION**

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

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

**Table 11 Ordering information for supported PCR plates**

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

# 3 Preparation of AMPure XP Bead Plates



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

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

# Step 1. Prepare the bead plate to be used for 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 library pools).

### Load the Agilent NGS Workstation

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

**Table 12 Initial Bravo deck configuration for AMPureXP_Aliquot (Concentration of Pool) protocol**

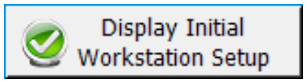
| Location | Content |
|---|---|
| 2 | New tip box |
| 5 | Empty Agilent Deep Well Plate |
| 6 | Reservoir of AMPure XP bead suspension prepared in **step 3** |
| 8 | Empty tip box |

### Run VWorks protocol AMPureXP_Aliquot (Second-Strand)

5 On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_Aliquot (Second-Strand)** protocol.

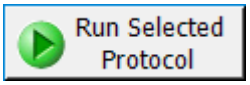
6 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

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 (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.

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 SecondStrand_XT_HS2_RNA protocol (refer to **Table 32** on page 61). Use the plate within the next 24 hours

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

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

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

### Prepare the 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

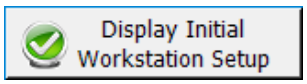
4. Load the Bravo deck according to **Table 13**.

**Table 13 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 |

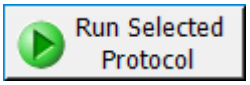
### Run VWorks protocol AMPureXP_Aliquot (Library Prep)

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 LibraryPrep_XT_HS2_ILM protocol (refer to **Table 40** on page 70). Use the plate within the next 24 hours.

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

# Step 3. Prepare the bead plate to be used for Pre-Capture Purification

The AMPureXP_XT_HS2_ILM (Pre-Capture PCR) protocols require a bead plate containing 50 µL of beads in each well. Use the AMPureXP_Aliquot (Pre-Capture PCR) protocol to prepare the bead plate needed for purification of pre-capture PCR products.

### Prepare the workstation and reagents for the AMPureXP_Aliquot (Pre-Capture PCR) protocol

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

### Load the Agilent NGS Workstation

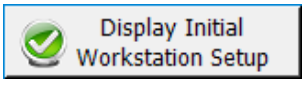
4. Load the Bravo deck according to **Table 14**.

**Table 14 Initial Bravo deck configuration for AMPureXP_Aliquot (Pre-Capture PCR) protocol**

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

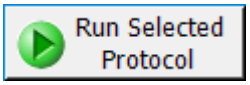
### Run VWorks protocol AMPureXP_Aliquot (Pre-Capture PCR)

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




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

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




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

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

11 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the AMPureXP_XT_HS2_ILM (Pre-Capture PCR) protocol (refer to **Table 51** on page 79). Use the plate within the next 24 hours.

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

# Step 4. Prepare the bead plate to be used for concentration of pooled DNA (pre-capture pooling workflow only)

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

The bead plate for the AMPureXP_XT_HS2_ILM (Concentration of Pool) protocol is only needed if you are running the pre-capture pooling workflow option. If you are using the post-capture pooling workflow, proceed to **"Step 5. Prepare the bead plate to be used for Post-Capture Purification"** on page 42.

### Prepare the workstation and reagents for the AMPureXP_Aliquot (Concentration of Pool) protocol

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

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

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

   **a** Verify that the AMPure XP bead suspension is at room temperature.

   **b** Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.

   **c** Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the AMPureXP_XT_HS2_ILM (Concentration of Pool) protocol (each column accommodates 8 DNA library pools).

### Load the Agilent NGS Workstation

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

**Table 15 Initial Bravo deck configuration for AMPureXP_Aliquot (Concentration of Pool) protocol**

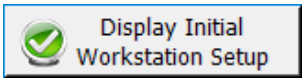
| Location | Content |
|---|---|
| 2 | New tip box |
| 5 | Empty Agilent Deep Well Plate |
| 6 | Reservoir of AMPure XP bead suspension prepared in **step 3** |
| 8 | Empty tip box |

### Run VWorks protocol AMPureXP_Aliquot (Concentration of Pool)

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

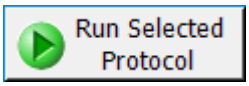
6 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

7 Click **Display Initial Workstation Setup**.




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

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




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

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

11 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the AMPureXP_XT_HS2_ILM (Concentration of Pool) protocol (refer to **Table 63** on page 99). Use the plate within the next 24 hours.

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

# Step 5. Prepare the bead plate to be used for Post-Capture Purification

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

### Prepare the workstation and reagents for the AMPureXP_Aliquot (Post-Capture PCR) protocol

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

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

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

- **a** Verify that the AMPure XP bead suspension is at room temperature.
- **b** Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
- **c** Directly pour the bead suspension from the bottle into the reservoir. Fill the columns of the reservoir with enough bead suspension to cover the pyramid-shaped wells. Only fill as many columns as will be needed for the AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocol (each column accommodates 8 indexed libraries).

### Load the Agilent NGS Workstation

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

**Table 16 Initial Bravo deck configuration for AMPureXP_Aliquot (Post-Capture PCR) protocol**

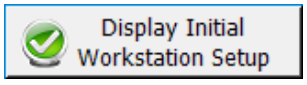
| Location | Content |
|---|---|
| 2 | New tip box |
| 5 | Empty Agilent Deep Well Plate |
| 6 | Reservoir of AMPure XP bead suspension prepared in **step 3** |
| 8 | Empty tip box |

### Run VWorks protocol AMPureXP_Aliquot (Post-Capture PCR)

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

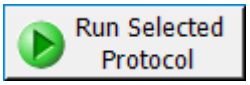
6 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

7 Click **Display Initial Workstation Setup**.




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

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




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

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

11 Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Keep at 4°C until needed in the AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocol (refer to **Table 92** on page 127). Use the plate within the next 24 hours.

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

# 4 Preparation of Input RNA and Conversion to cDNA



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

The protocol is compatible with both intact RNA prepared from fresh or fresh frozen samples and lower-quality RNA prepared from FFPE samples. For FFPE-derived RNA samples, begin the protocol using **"Step 1. Prepare RNA samples"** on page 46. For intact RNA samples, begin the protocol using **"Run the automated fragmentation protocol (both FFPE and intact RNA)"** on page 47.

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

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

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

**Table 17 Reagents thawed before use in protocol**

| Kit Component | Storage Location | Thawing Conditions | Mixing Method | Where Used |
|---|---|---|---|---|
| 2X Priming Buffer (tube with purple cap) | SureSelect cDNA Module (Pre PCR), –20°C | Thaw on ice then keep on ice | Vortexing | **page 48** |
| First Strand Master Mix (amber tube with amber cap)* | SureSelect cDNA Module (Pre PCR), –20°C | Thaw on ice for 30 minutes then keep on ice | Vortexing | **page 55** |
| Second Strand Enzyme Mix (bottle) | SureSelect cDNA Module (Pre PCR), –20°C | Thaw on ice then keep on ice | Vortexing | **page 59** |
| Second Strand Oligo Mix (tube with yellow cap) | SureSelect cDNA Module (Pre PCR), –20°C | Thaw on ice then keep on ice | Vortexing | **page 59** |

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

# Step 1. Prepare RNA samples

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

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

**NOTE**

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

If you must combine sample types in the same run — for example, when including a control sample of Agilent QPCR Human Reference Total RNA with a set of FFPE RNA samples — then the sample types need to be separated into separate plasticware at the end of the Fragmentation_XT_HS2_RNA protocol in order to accommodate the different processing steps (see **step 22** on **page 52**). Intact RNA samples should be transferred to a fresh PCR plate or tube before they are put in the thermal cycler to run the fragmentation program while the plate with the FFPE RNA samples is held on ice. At the end of the thermal cycling program, you can return the samples back to their original wells in the original PCR plate.

## Assess initial quality of RNA samples (FFPE RNA only)

The quality assessment is only needed for FFPE-derived total RNA samples. If you are using intact (non-FFPE) RNA samples, proceed directly to **"Run the automated fragmentation protocol (both FFPE and intact RNA)"** on page 47.

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

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

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

2. Examine the starting size distribution of RNA in the sample using one of the RNA qualification systems described in **Table 18**. Select the specific assay appropriate for your sample based on the RNA concentration determined in **step 1** on **page 46**.

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

**Table 18 RNA qualification platforms**

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

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

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

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

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

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

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

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

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

Intact total RNA samples (i.e., RNA prepared from fresh or fresh frozen samples or commercially-prepared reference RNA) require fragmentation prior to cDNA synthesis, while FFPE-derived RNA samples are already sufficiently fragmented and do not require further fragmentation. Importantly, however, FFPE RNA still needs to be run in the automated fragmentation protocol (Fragmentation_XT_HS2_RNA) in order to mix the samples with the 2X Priming Buffer, which includes both fragmentation agents and primers needed for cDNA synthesis in the following steps. At the end of the automated fragmentation protocol, intact RNA samples are transferred to a thermal cycler to be fragmented at 94°C (see thermal cycling program in **Table 20**). FFPE RNA samples are not subjected to this thermal cycling program.

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

### Prepare the workstation for protocol Fragmentation_XT_HS2_RNA (both FFPE and intact RNA)

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 (intact RNA only)

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

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

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

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

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

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

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

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

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

**Table 21 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 |

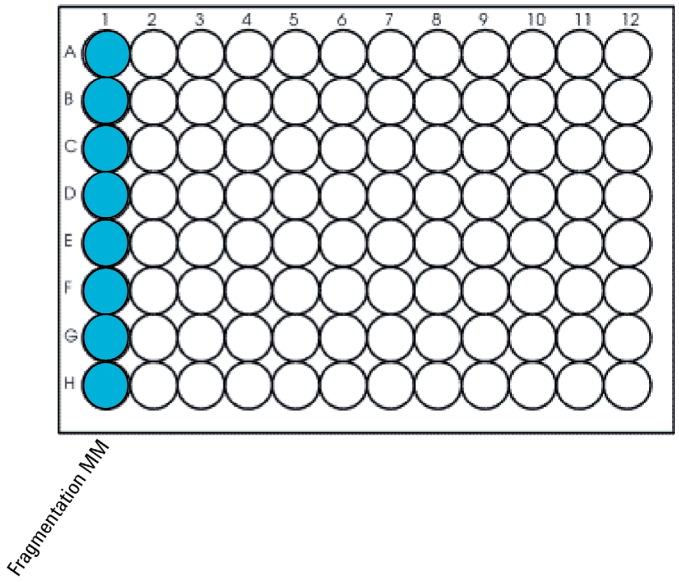



**Figure 3** Configuration of the **Agilent Deep Well** source plate for protocol Fragmentation_XT_HS2_RNA

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

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

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 (both FFPE and intact RNA)

**11** Load the Labware MiniHub according to **Table 22**, using the plate orientations shown in **Figure 4**.

**Table 22 Initial MiniHub configuration for Fragmentation_XT_HS2_RNA protocol**

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

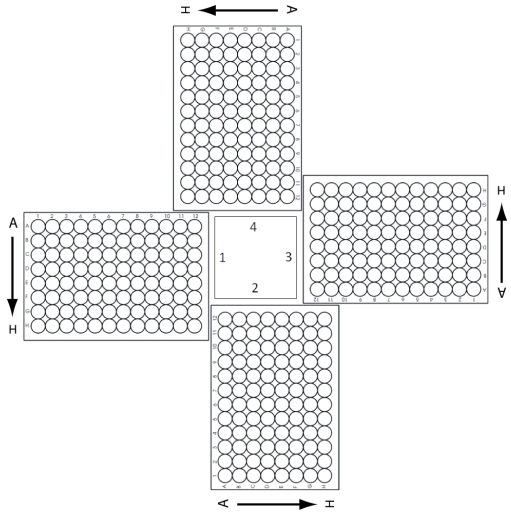



**Figure 4** Agilent Labware MiniHub plate orientation

**12** Load the Bravo deck according to **Table 23**.

**Table 23 Initial Bravo deck configuration for Fragmentation_XT_HS2_RNA protocol**

| Location | Content |
|---|---|
| 1 | Empty waste plate (Agilent 2 mL square well) |
| 5 | Empty Armadillo plate |
| 6 | RNA samples in 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 |

**13** Load the BenchCel Microplate Handling Workstation according to **Table 24**.

**Table 24 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 | — | — | — |

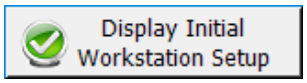
### Run VWorks protocol Fragmentation_XT_HS2_RNA (both FFPE and intact RNA)

**14** On the SureSelect setup form, under **Select protocol to execute**, select the **Fragmentation_XT_HS2_RNA** protocol.

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

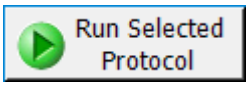
**16** Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

**17** Click **Display Initial Workstation Setup**.



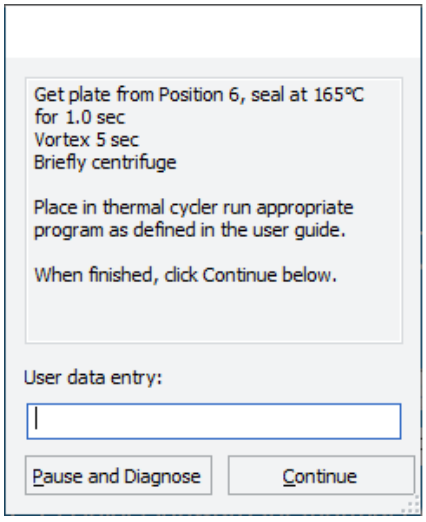

**18** Verify that the NGS workstation has been set up as displayed on the right side of the form.

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




Running the Fragmentation_XT_HS2_RNA protocol takes approximately 10 minutes. Once complete, the samples are ready for fragmentation (performed in the pre-programmed thermal cycler with intact RNA samples only). The samples are located in the PCR plate at position 6 of the Bravo deck.

**20** 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.



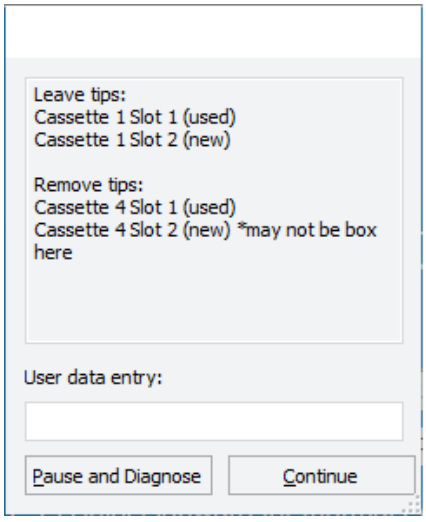

**21** Vortex the sealed plate at medium speed for 5 seconds, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

**22** Proceed as needed for your RNA sample type.

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

**23** In VWorks, click **Continue**.

The following prompt opens.




**24** 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_RNA protocol as described in **"Prepare the First Strand cDNA master mix source plate"** on page 55.

25 On the Labware MiniHub, leave the tip boxes in Cassette 1 in place. They will be used in the FirstStrandcDNA_XT_HS2_RNA protocol as described in **"Load the Agilent NGS Workstation"** on page 56.

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

# Step 2. Synthesize first strand cDNA

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

## Prepare the 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 a red PCR plate insert at Bravo deck position 6.

## Pre-program the thermal cycler for first strand cDNA synthesis

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

**Table 25 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 26**. 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 26 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) | 14.5 µL | 24.0 µL | 33.5 µL | 43.0 µL | 67.0 µL | 129.0 µL |

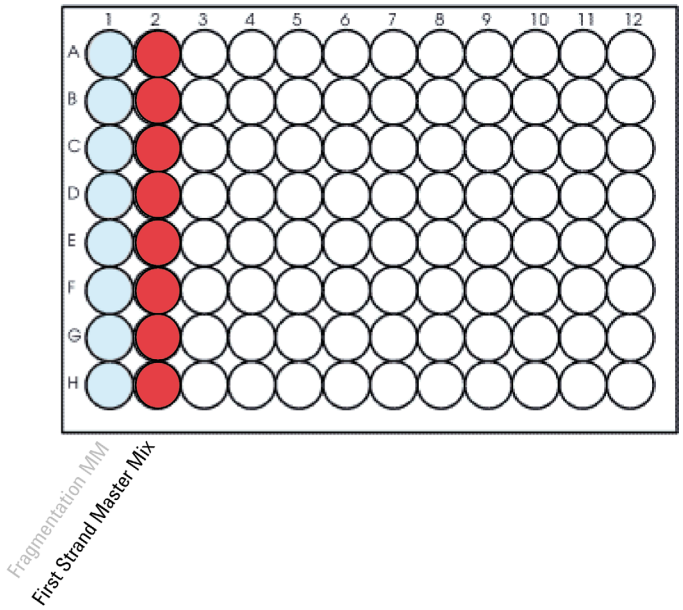



**Figure 5** Configuration of the **Agilent Deep Well** source plate for protocol FirstStrandcDNA_XT_HS2_RNA. The master mix dispensed during a previous protocol is shown in light shading.

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

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

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

## Load the Agilent NGS Workstation

1 Load the Labware MiniHub according to **Table 22**, using the plate orientations shown in **Figure 4** on page 50.

**Table 27 Initial MiniHub configuration for FirstStrandcDNA_XT_HS2_RNA 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)<br>**OR**<br>Empty tip box | — | — | Empty tip box |

2 Load the Bravo deck according to **Table 23**.

**Table 28 Initial Bravo deck configuration for FirstStrandcDNA_XT_HS2_RNA protocol**

| Location | Content |
|---|---|
| 1 | Empty waste plate (Agilent 2 mL square well) |
| 5 | Empty Armadillo plate |
| 6 | RNA sample plate (from **step 22** on **page 52**) seated in red insert (PCR plate type must be specified on setup form under Parameter 2) |
| 9 | First Strand cDNA master mix source plate, unsealed |

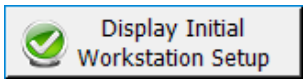
3 Load the BenchCel Microplate Handling Workstation according to **Table 24**.

**Table 29 Initial BenchCel configuration for FirstStrandcDNA_XT_HS2_RNA protocol**

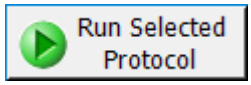
| No. of Columns Processed | Rack 1 | Rack 2 | Rack 3 | Rack 4 |
|---|---|---|---|---|
| 1 | 1 Tip box | — | — | — |
| 2 | 1 Tip box | — | — | — |
| 3 | 1 Tip box | — | — | — |
| 4 | 1 Tip box | — | — | — |
| 6 | 1 Tip box | — | — | — |
| 12 | 1 Tip box | — | — | — |

## Run VWorks protocol FirstStrandcDNA_XT_HS2_RNA

1. On the SureSelect setup form, under **Select protocol to execute**, select the **FirstStrandcDNA_XT_HS2_RNA** protocol.
2. Under **Select labware for thermal cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.
3. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
4. Click **Display Initial Workstation Setup**.

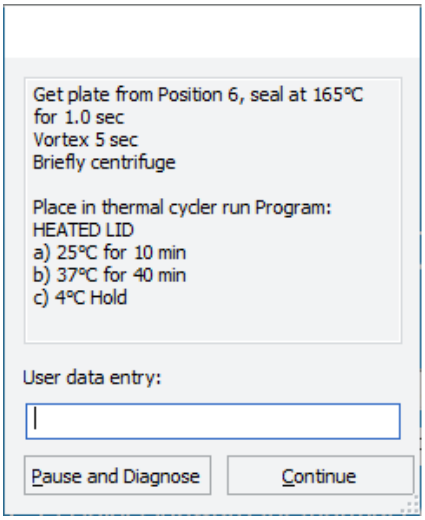



5. Verify that the NGS workstation has been set up as displayed on the right side of the form.
6. When verification is complete, click **Run Selected Protocol**.




Running the FirstStrandcDNA_XT_HS2_RNA protocol takes approximately 10 minutes. Once complete, the samples are ready for first-strand cDNA synthesis (performed in the preprogrammed thermal cycler). The samples are located in the PCR plate at position 6 of the Bravo deck.

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

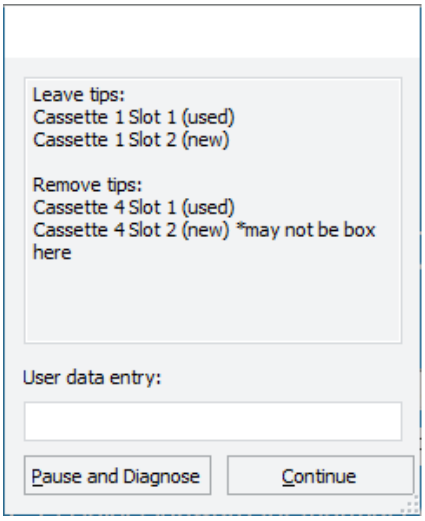



8 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

9 Immediately place the sample plate in the thermal block. Close the lid and resume the thermal cycler program in **Table 25**.

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 59.

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 61.

13 Once the thermal cycler program in **Table 25** reaches the 4°C Hold step, transfer the first strand cDNA sample plate from the thermal cycler to ice or a cold block. Proceed immediately to **"Step 3. Synthesize and purify second strand cDNA"** on page 59.

# Step 3. Synthesize and purify second strand cDNA

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.

This step uses the aliquoted plate of AMPure XP beads that was prepared on **page 34**.

## Prepare the workstation for runset SecondStrandcDNA_XT_HS2_RNA

1. Leave the tip boxes that were used in Cassette 1 of the Labware MiniHub during the FirstStrandcDNA_XT_HS2_RNA 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 14°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
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 a red PCR plate insert at Bravo deck position 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 30** 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 30 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 31**. 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 31 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 |

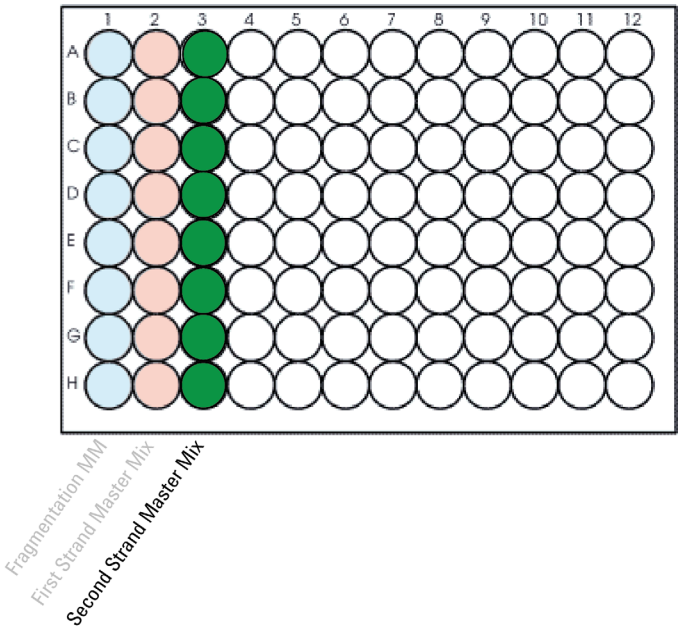



**Figure 6** Configuration of the **Agilent Deep Well** source plate for runset SecondStrandcDNA_XT_HS2_RNA. The master mixes dispensed during previous protocols are shown in light shading.

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

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

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

## 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 LibraryPrep_XT_HS2_ILM 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 32**, using the plate orientations shown in **Figure 4** on page 50.

**Table 32 Initial MiniHub configuration for SecondStrandcDNA_XT_HS2_RNA runset**

| Vertical Shelf Position | Cassette 1 | Cassette 2 | Cassette 3 | Cassette 4 |
|---|---|---|---|---|
| Shelf 5 (Top) | — | Aliquotted AMPure XP beads in Agilent deep well plate from **page 34** | — | — |
| Shelf 4 | — | — | — | — |
| Shelf 3 | — | Empty 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)<br>**OR**<br>Empty tip box | 70% ethanol reservoir from **step 2** | — | Empty tip box |

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

**Table 33 Initial Bravo deck configuration for SecondStrandcDNA_XT_HS2_RNA runset**

| Location | Content |
|---|---|
| 1 | Empty waste plate (Agilent 2 mL square well) |
| 5 | Empty 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 34**.

**Table 34 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 | — | — | — |

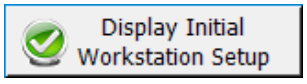
## Run VWorks runset SecondStrandcDNA_XT_HS2_RNA

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

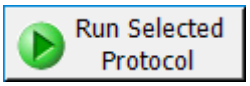
3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

4 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 SecondStrandcDNA_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 Armadillo 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 LibraryPrep_XT_HS2_ILM runset (see **"Load the Agilent NGS Workstation"** on page 70).

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 LibraryPrep_XT_HS2_LIM protocol as described in **"Prepare the master mix source plate"** on page 68.

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

# 5 Library Preparation



This chapter 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 target enrichment workflow, see **Figure 2** on page 27.

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

# Step 1. Prepare adaptor-ligated libraries

In this step, the Agilent NGS Workstation completes the DNA end modification steps required for SureSelect target enrichment, 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.

This step uses the components listed in **Table 35**. Thaw and mix each component as directed in **Table 35** before use. Before starting the run, you need to prepare master mixes (with overage) for each step, without the DNA sample. Master mixes for runs that include 1, 2, 3, 4, 6, and 12 columns (including overage) are shown in reagent preparation tables.

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

**Table 35 Reagents thawed before use in the protocol LibraryPrep_XT_HS2_ILM**

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

## Prepare the workstation

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

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

1 Prepare the appropriate volume of End Repair/dA-Tailing master mix, using volumes listed in **Table 36** 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 36 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 | 884 µL | 1836 µL |
| End Repair-A Tailing Enzyme Mix (tube with orange cap) | 4 µL | 51 µL | 85 µL | 119 µL | 153 µL | 221 µL | 459 µL |
| **Total Volume** | **20 µL** | **255 µL** | **425 µL** | **595 µL** | **765 µL** | **1105 µL** | **2295 µL** |

## Prepare the Ligation master mix

1 Prepare the appropriate volume of Ligation master mix, using volumes listed in **Table 37** 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 37 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 | 1270.8 µL | 2737 µL |
| T4 DNA Ligase (tube with blue cap) | 2 µL | 25.5 µL | 42.5 µL | 59.5 µL | 76.5 µL | 110.5 µL | 238 µL |
| **Total Volume** | **25 µL** | **318.8 µL** | **531.3 µL** | **743.8 µL** | **956.3 µL** | **1381.3 µL** | **2975 µL** |

## Prepare the Adaptor Oligo Mix

1 Prepare the appropriate volume of Adaptor Oligo Mix dilution, according to **Table 38**. Mix well using a vortex mixer and keep on ice.

**Table 38 Preparation of Adaptor Oligo Mix dilution**

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

## Prepare the 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 39** 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 39 Preparation of the master mix source plate for LibraryPrep_XT_HS2_ILM runset**

| Master Mix Solution | Position on Source Plate | Volume of master mix added per Well of Agilent Deep Well Source Plate | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1-Column Runs | 2-Column Runs | 3-Column Runs | 4-Column Runs | 6-Column Runs | 12-Column Runs |
| End Repair-dA Tailing master mix | Column 4 (A4-H4) | 31.0 µL | 52.0 µL | 73.0 µL | 94.0 µL | 136.0 µL | 280.0 µL |
| Ligation master mix | Column 5 (A5-H5) | 36.0 µL | 62.0 µL | 88.0 µL | 114.0 µL | 166.0 µL | 360.0 µL |
| Adaptor Oligo Mix dilution | Column 6 (A6-H6) | 15.0 µL | 22.5 µL | 30.0 µL | 37.5 µL | 52.5 µL | 101.3 µL |

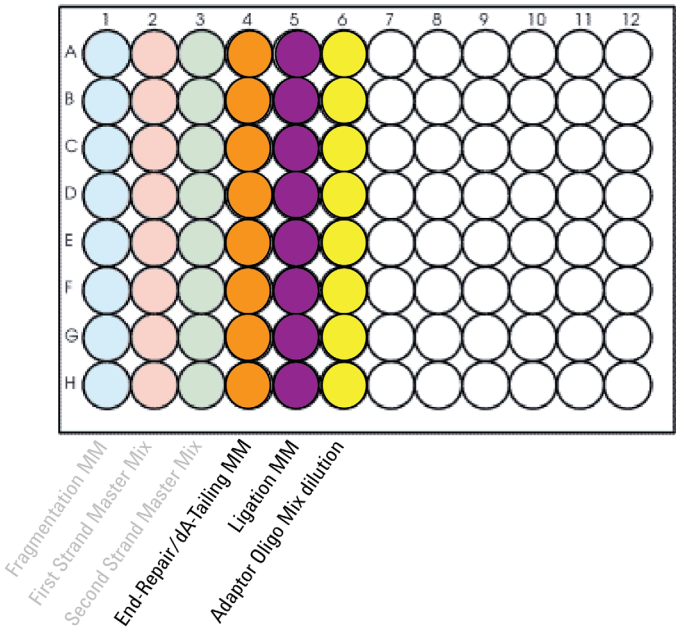



**Figure 7** Configuration of the **Agilent Deep Well** master mix source plate for runset LibraryPrep_XT_HS2_ILM. The master mixes dispensed during previous protocols are shown in light shading.

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

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

NOTE

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

## 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 (Pre-Cap PCR) protocol.

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 40**, using the plate orientations shown in **Figure 4** on page 50.

**Table 40 Initial MiniHub configuration for LibraryPrep_XT_HS2_ILM runset**

| Vertical Shelf Position | Cassette 1 | Cassette 2 | Cassette 3 | Cassette 4 |
|---|---|---|---|---|
| Shelf 5 (Top) | Aliquotted AMPure XP beads in Agilent deep well plate from **page 36** | — | — | — |
| Shelf 4 | Empty Armadillo plate | — | — | — |
| Shelf 3 | Empty Armadillo plate | Empty 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)<br>**OR**<br>Empty tip box | 70% ethanol reservoir from **step 2** | — | Empty tip box |

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

**Table 41 Initial Bravo deck configuration for LibraryPrep_XT_HS2_ILM runset**

| Location | Content |
|---|---|
| 1 | Empty waste plate (Agilent 2 mL square well) |
| 4 | Empty red insert |
| 6 | Empty Armadillo plate |
| 7 | Armadillo plate containing cDNA samples |
| 9 | Library Prep master mix source plate, unsealed |

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

**Table 42 Initial BenchCel configuration for LibraryPrep_XT_HS2_ILM runset**

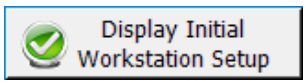
| No. of Columns Processed | Rack 1 | Rack 2 | Rack 3 | Rack 4 |
|---|---|---|---|---|
| 1 | 1 Tip box | — | — | — |
| 2 | 2 Tip boxes | — | — | — |
| 3 | 2 Tip boxes | — | — | — |
| 4 | 3 Tip boxes | — | — | — |
| 6 | 4 Tip boxes | — | — | — |
| 12 | 7 Tip boxes | — | — | — |

### Run VWorks runset LibraryPrep_XT_HS2_ILM

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

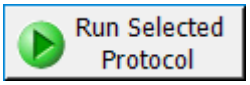
5 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

6 Click **Display Initial Workstation Setup**.

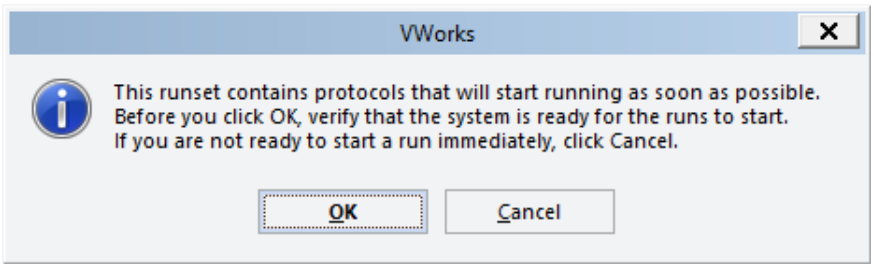



7 Verify that the NGS workstation has been set up as displayed on the right side of the form.

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




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




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

# Step 2. Amplify adaptor-ligated libraries

In this step, the Agilent NGS Workstation completes the liquid handling steps for 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.

This step uses the components listed in **Table 43**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

**Table 43 Reagents for pre-capture PCR amplification**

| Component | Storage Location | Mixing Method | Where Used |
|---|---|---|---|
| Herculase II Fusion DNA Polymerase (tube with red cap) | SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C | Pipette up and down 15–20 times | **page 74** |
| 5× Herculase II Reaction Buffer (tube with clear cap) | SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR), –20°C | Vortexing | **page 74** |
| SureSelect XT HS2 Index Primer Pairs | SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR),* –20°C | Vortexing | **page 74** |

* 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**. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.

## Pre-program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 44**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the automation run.

**Table 44 Pre-Capture PCR Thermal Cycler Program***

| Segment | Number of Cycles | Temperature | Time |
|---|---|---|---|
| 1 | 1 | 98°C | 2 minutes |
| 2 | 10 to 14 (see **Table 45** 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 45 Pre-capture PCR cycle number recommendations**

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

## Prepare the SureSelect XT HS2 Index Primer Pairs

1 Using a multichannel pipette, transfer 5 µL of each SureSelect XT HS2 Index Primer Pair from the 96-well plate in which the primer pairs are provided into the PCR plate to be used for the pre-capture PCR thermal cycling. Make sure to maintain the same well location for each primer pair when transferring to the PCR plate. Keep the PCR plate on ice.

The PCR plate containing the primer pairs is loaded onto the Bravo deck in **step 2** on **page 75** for the Pre-CapPCR_XT_HS2_ILM protocol.

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

1 Prepare the appropriate volume of pre-capture PCR master mix, according to **Table 46**. Vortex at medium speed for 15–20 seconds and keep on ice.

**Table 46 Preparation of Pre-Capture PCR Master Mix**

| Reagent | Volume for 1 Library | Volume for 1 Column | Volume for 2 Columns | Volume for 3 Columns | Volume for 4 Columns | Volume for 6 Columns | Volume for 12 Columns |
|---|---|---|---|---|---|---|---|
| 5× Herculase II Buffer with dNTPs (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 an **Armadillo** master mix source plate, add the volume of PCR master mix indicated in **Table 47** to all wells of column 1 of the master mix source plate. The final configuration of the master mix source plate is shown in **Figure 8**.

**Table 47 Preparation of the master mix source plate for Pre-CapPCR_XT_HS2_ILM protocol**

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

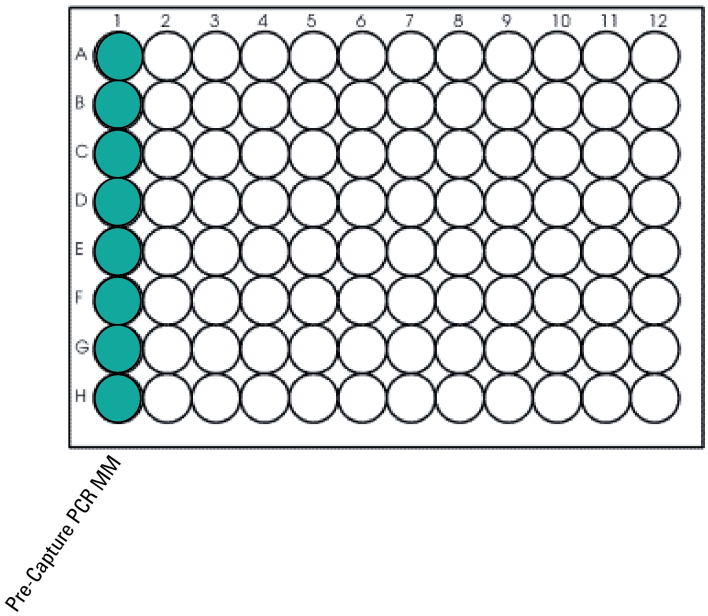



**Figure 8** Configuration of the **Armadillo** master mix source plate for protocol Pre-CapPCR_XT_HS2_ILM

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

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

NOTE

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

## Load the Agilent NGS Workstation

1 Load the Labware MiniHub according to **Table 48**, using the plate orientations shown in **Figure 4** on page 50.

**Table 48 Initial MiniHub configuration for Pre-CapPCR_XT_HS2_ILM protocol**

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

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

**Table 49 Initial Bravo deck configuration for Pre-CapPCR_XT_HS2_ILM protocol**

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

3 Load the BenchCel Microplate Handling Workstation according to **Table 50**.

**Table 50 Initial BenchCel configuration for Pre-CapPCR_XT_HS2_ILM protocol**

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

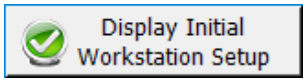
### Run VWorks protocol Pre-CapPCR_XT_HS2_ILM

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

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

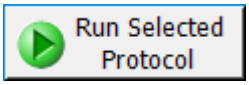
6 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

7 Click **Display Initial Workstation Setup**.



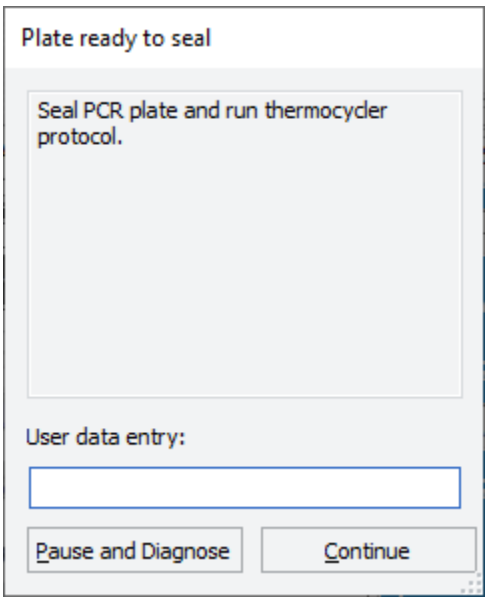

8 Verify that the NGS workstation has been set up as displayed on the right side of the form.

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




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

10 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.




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

12 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 44**. 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

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.

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

## Prepare the workstation and reagents

1. Retain the Armadillo master mix source plate containing the Pre-Capture PCR master mix located at position 9 of the Bravo deck for later use in the TS_D1000 protocol (see **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape"** on page 81). Otherwise, clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 45°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
5. Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water. Use the same Agilent shallow well reservoir that was used in the LibraryPrep_XT_HS2_ILM runset.

   Make sure that the water in the reservoir does not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.
6. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.
7. Load the Labware MiniHub according to **Table 51**, using the plate orientations shown in **Figure 4** on page 50.

**Table 51 Initial MiniHub configuration for AMPureXP_XT_HS2_ILM (Pre-Capture PCR) protocol**

| Vertical Shelf Position | Cassette 1 | Cassette 2 | Cassette 3 | Cassette 4 |
|---|---|---|---|---|
| Shelf 5 (Top) | Aliquotted AMPure XP beads in Agilent Deep Well plate from **page 38** | — | — | — |
| Shelf 4 | — | — | — | — |
| Shelf 3 | — | Empty Armadillo Plate | — | — |
| Shelf 2 | — | Nuclease-free water reservoir from **step 5** | | — |
| Shelf 1 (Bottom) | — | 70% ethanol reservoir from **step 6** | — | Empty tip box |

**8** Load the Bravo deck according to **Table 52**.

**Table 52 Initial Bravo deck configuration for AMPureXP_XT_HS2_ILM (Pre-Capture PCR) protocol**

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

**9** Load the BenchCel Microplate Handling Workstation according to **Table 53**.

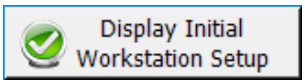
**Table 53 Initial BenchCel configuration for AMPureXP_XT_HS2_ILM (Pre-Capture PCR) protocol**

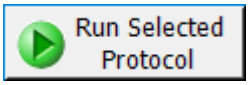
| No. of Columns Processed | Rack 1 | Rack 2 | Rack 3 | Rack 4 |
|---|---|---|---|---|
| 1 | 1 Tip box | — | — | — |
| 2 | 1 Tip box | — | — | — |
| 3 | 2 Tip boxes | — | — | — |
| 4 | 2 Tip boxes | — | — | — |
| 6 | 3 Tip boxes | — | — | — |
| 12 | 5 Tip boxes | — | — | — |

## Run the appropriate *AMPureXP_XT_HS2_ILM (Pre-Cap PCR)* protocol

1. On the SureSelect setup form, under **Select protocol to execute**, select one of the AMPureXP_XT_HS2_ILM (Pre-Capture PCR) protocols based on your workflow of choice.
   - If you are using the post-capture pooling workflow (i.e., pooling samples after hybridization with the Probe) then select the protocol **AMPureXP_XT_HS2_ILM (Pre-Cap PCR - SinglePlex)**.
   - If you are using the pre-capture pooling workflow (i.e., pooling samples prior to hybridization with the Probe) then select the protocol **AMPureXP_XT_HS2_ILM (Pre-Cap PCR - MultiPlex)**.

   The single-plex and multi-plex protocols use different elution volumes. Selecting the correct option is important for the downstream hybridization protocol.
2. Under **Select labware for thermal cycling**, select the specific type of PCR plate containing the amplified libraries at position 9.
3. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
4. Click **Display Initial Workstation Setup**.

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

   The purification protocol takes approximately 45 minutes. When complete, the purified DNA samples are in the Armadillo plate located on Bravo deck position 7.
7. 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 81.
- 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 85.

## 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 **Armadillo** source plate that was used in the Pre-CapPCR_XT_HS2_ILM protocols, prepare the Sample Buffer source plate at room temperature. Add the volume of D1000 Sample Buffer indicated in **Table 54** to each well of column 2 of the plate.

**Table 54 Preparation of the Sample Buffer source plate for TS_D1000 protocol**

| Solution | Position on Source Plate | Volume of Sample Buffer added per Well of Armadillo 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.

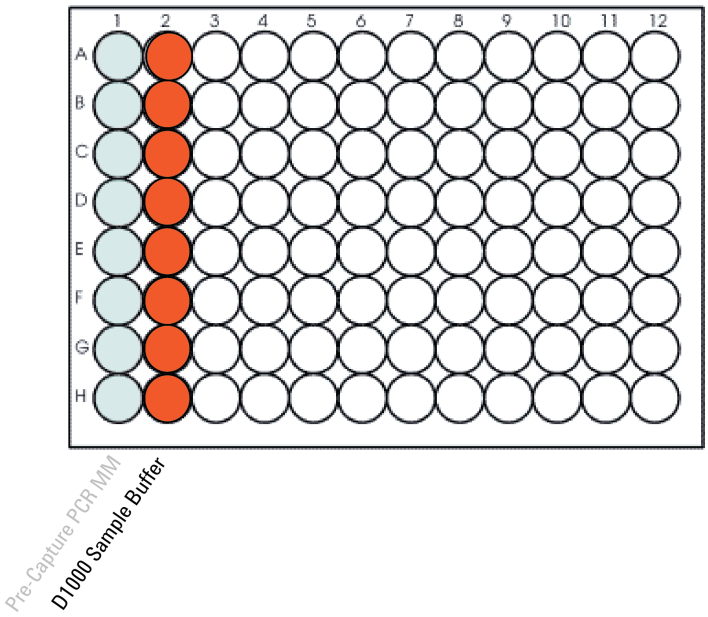



**Figure 9** Configuration of the **Armadillo** source plate for protocol TS_D1000. The master mix dispensed during a previous protocol is shown in light shading.

### Load the Agilent NGS Workstation

6 Load the Labware MiniHub according to **Table 55**, using the plate orientations shown in **Figure 4** on page 50.

**Table 55 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 56**.

**Table 56 Initial Bravo deck configuration for TS_D1000 protocol**

| Location | Content |
|---|---|
| 4 | Amplified pre-capture libraries in Armadillo plate (unsealed) |

**Table 56 Initial Bravo deck configuration for TS_D1000 protocol**

| Location | Content |
|---|---|
| 6 | Empty TapeStation analysis plate (Agilent p/n 5042-8502) |
| 9 | Armadillo source plate containing D1000 Sample Buffer in Column 3 |

**CAUTION**

To prevent damage to the Agilent 4200 TapeStation instrument and the Agilent NGS Workstation, use only the specified Agilent plates (Agilent p/n 95042-8502) 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 57**.

**Table 57 Initial BenchCel configuration for TS_D1000 protocol**

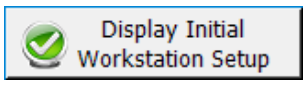
| No. of Columns Processed | Rack 1 | Rack 2 | Rack 3 | Rack 4 |
|---|---|---|---|---|
| 1 | 1 Tip box | — | — | — |
| 2 | 1 Tip box | — | — | — |
| 3 | 1 Tip box | — | — | — |
| 4 | 1 Tip box | — | — | — |
| 6 | 1 Tip box | — | — | — |
| 12 | 1 Tip box | — | — | — |

### Run VWorks protocol TS_D1000

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

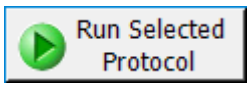
**10** Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

**11** Click **Display Initial Workstation Setup**.



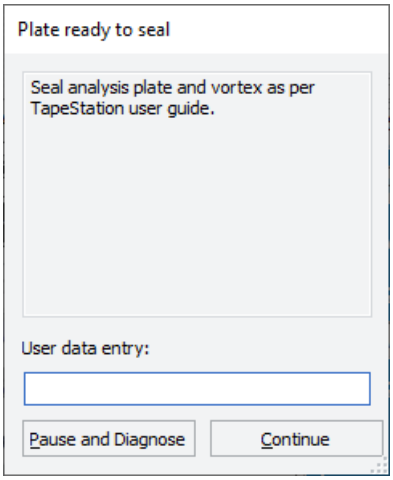

**12** Verify that the NGS workstation has been set up as displayed on the right side of the form.

**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 until the samples are used for hybridization set up on **page 89**.

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 58** for guidelines). Sample electropherograms are shown in **Figure 10** (library prepared from high-quality DNA) and **Figure 11** (library prepared from medium-quality FFPE DNA).

The appearance of an additional low molecular weight peak indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to that seen in the example electropherogram in **Figure 11**. See Troubleshooting information on **page 169** for additional considerations.

**Table 58 Pre-capture library qualification guidelines**

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

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

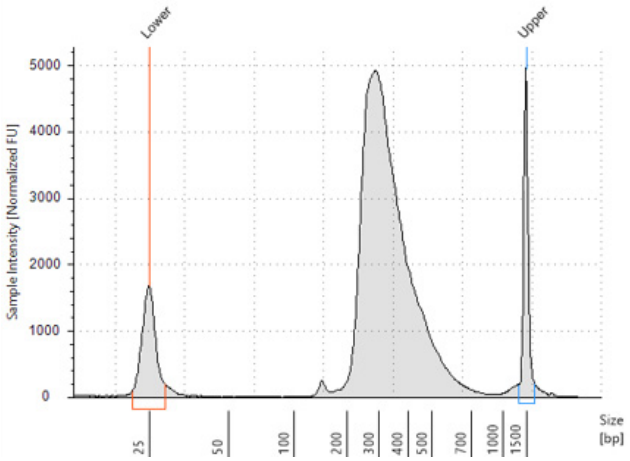



**Figure 10** Pre-capture library prepared from high-quality RNA sample (Human Reference Total RNA) analyzed using a D1000 ScreenTape assay

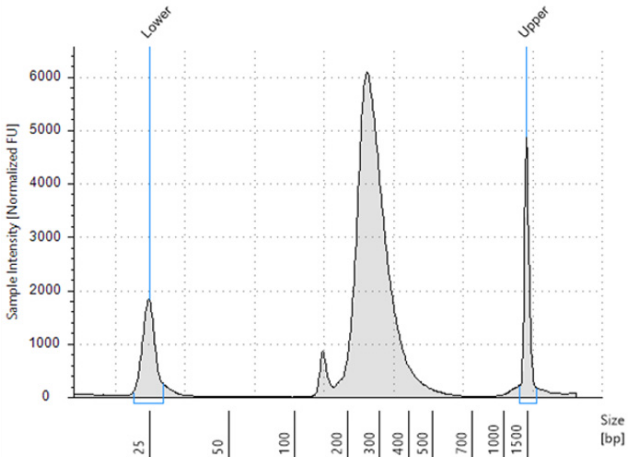



**Figure 11** Pre-capture library prepared from a typical FFPE RNA sample analyzed using a D1000 ScreenTape assay

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

## Option 2: Analysis using an equivalent platform (non-automated)

Using manual preparation of the analytical sample plate, you can analyze the 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** through **Figure 11**). **Table 59**. Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 58** for guidelines). **Table 59** includes links to assay instruction.

**Table 59 Pre-capture library analysis options**

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

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

# 6 Hybridization



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

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

- If you are pooling samples after hybridization to the Probe, then follow the steps in **"Step 1, Option 1. Prepare DNA for Single-Plex Hybridization"** on page 88.
- If you are pooling samples prior to hybridization to the Probe, then follow the steps in **"Step 1, Option 2. Prepare DNA for Multi-Plex Hybridization"** on page 91.

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

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

Follow the steps in this section if you are using the post-capture pooling workflow. If you are using the pre-capture pooling workflow, see **"Step 1, Option 2. Prepare DNA for Multi-Plex Hybridization"** on page 91.

## Aliquot prepped DNA samples for hybridization

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

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

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

Using the DNA concentration for each sample determined on **page 81** to **page 85**, calculate the volume of each sample to be used for hybridization using the formula below:

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

If the concentration of any sample is not sufficient to allow use of the recommended 400 ng of DNA, use the full remaining volume of DNA sample (approximately 10 to 12 µL, containing at least 200 ng) and manually prepare the sample plate for the hybridization step rather than using the Aliquot_Libraries automation protocol. In such cases, there is only enough DNA sample for one hybridization reaction instead of two.

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

1. Create a .csv (comma separated value) file with the headers shown in **Figure 12**. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in .csv format. The file must include rows for all 96 wells of the plate.
2. Enter the information requested in the header for each DNA sample.
   - In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
   - In the SourceWell and DestinationWell fields, enter each well position for the plate. SourceWell and DestinationWell field contents must be identical for a given sample.
   - In the Volume field, enter the volume (in µL) of each DNA sample to be used in the hybridization step (see **page 88** for guidelines). For all empty wells on the plate, delete the corresponding rows in the .csv file.

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

**Figure 12** Sample spreadsheet for 1-column run

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_RNA_ILM_v.Bx.x.x\Aliquot Library Input Files\Aliquot_Libraries_Template.csv** (where x.x.x is the version number).

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

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_RNA_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.

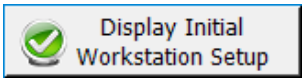
5 Load the Bravo deck according to **Table 60**.

**Table 60 Initial Bravo deck configuration for Aliquot_Libraries protocol**

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

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

7 Click **Display Initial Workstation Setup**.



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

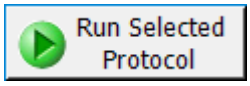
**a** Click the "**...**" button below **Select Aliquot Input File** to open a directory browser window.


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

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

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

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




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

11 Remove the sample plate from the Bravo deck.

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

Follow the steps in this section if you are using the pre-capture pooling workflow. If you are using the post-capture pooling workflow, see **"Step 1, Option 1. Prepare DNA for Single-Plex Hybridization"** on page 88.

## Pool indexed DNA samples for hybridization

In this step, the workstation pools the prepped indexed cDNA samples before hybridization to the Probe. This workflow step is set up using the VWorks Form XT_HS2_Pooling.VWForm shown below, which is accessible from within the XT_HS2_RNA_ILM form.


### Plan pooling run parameters

The hybridization reaction requires 600 ng indexed cDNA (or 1200 ng for the SureSelect XT HS PreCap Human All Exon V8/V8+UTR Probes), made up of a pool containing equal amounts of 8 or 16 individual libraries. See **Table 61** for the recommended pool composition based on your SureSelect or ClearSeq Probe.

**Table 61 Pre-capture pooling of indexed DNA libraries**

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

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

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

Accurate normalization of pools requires a minimum pipetting volume of 2 µL for each sample. Maximum DNA concentration values for a pool containing >2 µL of each sample are shown in **Table 61**, above. When higher-concentration DNA samples are included in the pooling run, the DNA pool amount must be adjusted as described below.

- Check the DNA concentration of each sample in the set of source plates to be pooled to a single destination plate to determine the appropriate amount of DNA per pool.
  - If all samples contain DNA at concentrations below the maximum DNA concentration shown in **Table 61**, then prepare 1200 ng DNA pools (or a 2400 ng pool if using SureSelect XT HS PreCap Human All Exon V8 Probe).
  - If at least one of the samples is above the maximum DNA concentration shown in **Table 61**, then you need to calculate the appropriate DNA pool amount. First, identify the most concentrated DNA sample and calculate the amount of DNA contained in 2 µL of that sample. This becomes the amount of each DNA sample used for pooling in the run. For example, if the highest DNA sample concentration is 200 ng/µL, then the final DNA pool will contain 400 ng of each indexed DNA. Next, determine the total amount of DNA per pool, based on the Probe size. Continuing with the same example, a Focused Exome capture pool would contain 8 × 400 ng, or 3200 ng DNA.

### Plan destination indexed DNA pool sample plate configuration

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

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

### Prepare .csv files for pooling and normalization

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

See **Figure 13** for required .csv file content. Pooling and normalization .csv file templates are provided in the following directory:

**C:\VWorks Workspace\NGS Option B\XT_HS2_RNA_ILM_v.Bx.x.x\Pooling and Normalization Templates**

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

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

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

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

**Figure 13** Sample pooling and normalization .csv file content

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

- In the **PreCap Amplified pond concentrations** field, enter the concentration (in ng/µL) determined on **page 81** for each indexed DNA sample.
- In the **Target WellID** field, enter the well position of the pool in which the indexed DNA sample should be included for the destination plate. See the guidelines on **page 92** for hybridization sample pool placement considerations.

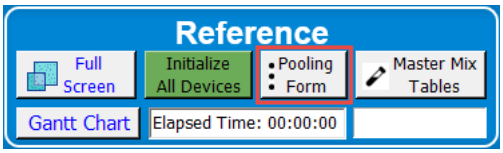
### Set up and run the PreCapture_Pooling automation protocol

14 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.

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

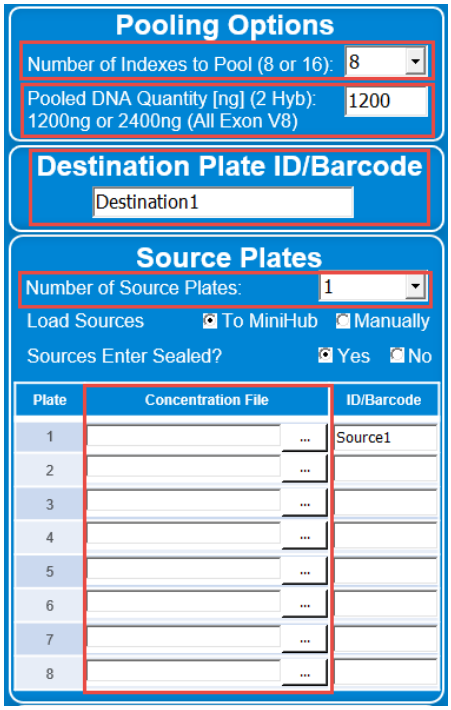
16 To set up the PreCapture_Pooling automation protocol, open the VWorks Form XT2_HS2_Pooling using one of the methods below.

- Double-click the shortcut on your desktop for the XT2_HS2_Pooling VWorks Form.
- In the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_RNA_ILM_v.Bx.x.x\Forms** (where x.x.x is the version number) open the file **XT_HS2_Pooling_v.Bx.x.x.VWForm**.
- From the XT_HS2_RNA_ILM VWorks Form, under **Reference**, click **Pooling Form.**




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

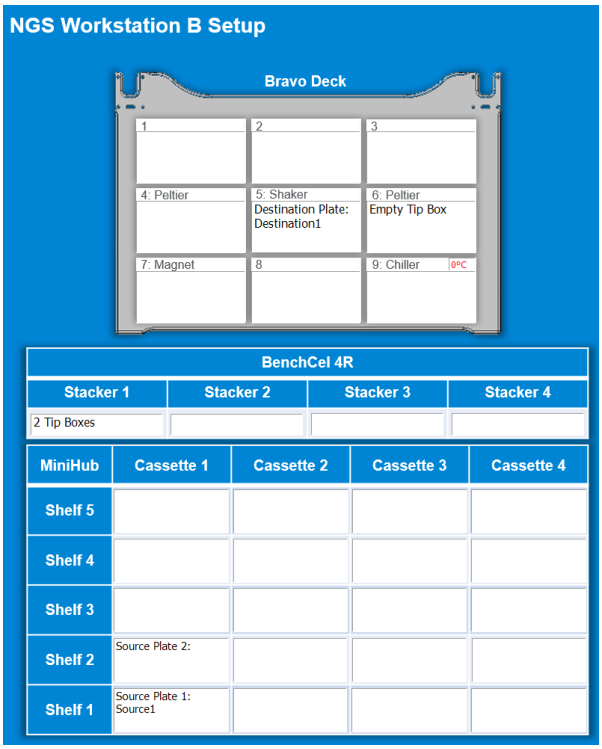
- Under **Pooling Options**, from **Number of Indexes to Pool** menu, select 8 or 16 (see **Table 61** on page 92 for guidelines).
- Under **Pooling Options**, from **Pooled DNA Quantity** menu, enter the required total amount of DNA in the pool.
  - For SureSelect XT HS Human All Exon V8 Probe, the required amount is 2400 ng. For all other Probes, the typical amount is 1200 ng. These amounts are sufficient for two hybridization reactions. See **page 91** for guidelines.
- Under **Destination Plate ID/Barcode**, enter the name or barcode of the destination plate into the field provided.
- Under **Source Plates**, from **Number of Source Plates** menu, select the number of indexed DNA source plates to be provided for sample pooling. If >8 plates will be used to create a single hybridization sample plate, run the pooling and normalization protocol in sets of 8 source plates.
- Under **Source Plates**, specify whether the indexed DNA source plates will be loaded in the MiniHub and will be sealed at start of run (recommended).
- In the table under **Source Plates**, in **Concentration File** field, use the browse button to specify the location of each .csv file that provides sample position and concentration data for each plate.



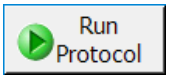

**18** When finished entering run parameters in the Form, click **Display Setup**.

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

- Load each indexed DNA source plate onto its assigned shelf on the MiniHub.
- Load an Armadillo destination plate on Bravo deck position 5.
- Load an empty tip box on Bravo deck position 6.
- Load the indicated number of tip boxes in BenchCel Stacker 1.




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




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

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

**21** Remove the destination plate containing the indexed DNA pool samples from Bravo deck position 5.

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

# Adjust final concentration of pooled DNA

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

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

### Prepare .csv file for normalizing sample volumes to 100 µL

1 Create a .csv (comma separated value) file with the headers shown in **Figure 14**. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in .csv format. The file must include rows for all 96 wells of the plate.

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

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

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

**Figure 14** Sample Aliquot_Water .csv file content

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_RNA_ILM_v.Bx.x.x\Aliquot Library Input Files** (where x.x.x is the version number).

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

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

### Set up and run the Aliquot_Water automation protocol

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

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

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

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

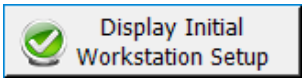
6 Load the Bravo deck according to **Table 62**.

**Table 62 Initial Bravo deck configuration for Aliquot_Water protocol**

| Location | Content |
|---|---|
| 5 | Pooled and normalized library cDNA in Armadillo plate |
| 6 | Empty tip box |
| 8 | New tip box |
| 9 | Nuclease-free water reservoir from **step 5** |

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

8 Click **Display Initial Workstation Setup**.




9 Upload the .csv file created in **"Prepare .csv file for normalizing sample volumes to 100 µL"** on page 97.

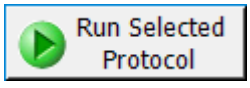
**a** Click the browse button ("**...**") below **Select Aliquot Input File** to open a directory browser window.


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

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

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

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




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

12 Remove the sample plate from the Bravo deck position 5.

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

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

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

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

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

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

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

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

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

20 Load the Labware MiniHub according to **Table 63**, using the plate orientations shown in **Figure 4** on page 50.

**Table 63 Initial MiniHub configuration for AMPureXP_XT_HS2_ILM (Concentration of Pool) protocol**

| Vertical Shelf Position | Cassette 1 | Cassette 2 | Cassette 3 | Cassette 4 |
|---|---|---|---|---|
| Shelf 5 (Top) | Aliquotted AMPure XP beads in Agilent deep well plate from **page 40** | — | — | — |
| Shelf 4 | — | — | — | — |
| Shelf 3 | — | Empty Armadillo Plate | — | — |
| Shelf 2 | — | Nuclease-free water reservoir from **step 18** | | — |
| Shelf 1 (Bottom) | — | 70% ethanol reservoir from **step 19** | — | Empty tip box |

21 Load the Bravo deck according to **Table 64**.

**Table 64 Initial Bravo deck configuration for AMPureXP_XT_HS2_ILM (Concentration of Pool) protocol**

| Location | Content |
|---|---|
| 1 | Empty waste plate (Agilent 2 mL square well) |
| 9 | Armadillo plate containing DNA library pools from the Aliquot_Water protocol |

**22** Load the BenchCel Microplate Handling Workstation according to **Table 65**.

**Table 65 Initial BenchCel configuration for AMPureXP_XT_HS2_ILM (Concentration of Pool) protocol**

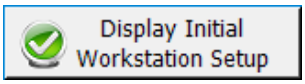
| No. of Columns Processed | Rack 1 | Rack 2 | Rack 3 | Rack 4 |
|---|---|---|---|---|
| 1 | 1 Tip box | — | — | — |
| 2 | 1 Tip box | — | — | — |
| 3 | 2 Tip boxes | — | — | — |
| 4 | 2 Tip boxes | — | — | — |
| 6 | 3 Tip boxes | — | — | — |
| 12 | 5 Tip boxes | — | — | — |

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

**24** Under **Select labware for thermal cycling**, select **96 Armadillo PCR in Red Alum Insert**.

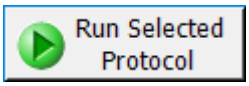
**25** Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

**26** Click **Display Initial Workstation Setup**.




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

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




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

# Step 2. Hybridize the cDNA library or library pool and Probe

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

This step uses the components listed in **Table 66**. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

**Table 66 Reagents for Hybridization**

| Kit Component | Storage Location | Thawing Conditions | Where Used |
|---|---|---|---|
| SureSelect XT HS2 Blocker Mix (blue cap) | SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C | Thaw on ice | **page 104** |
| SureSelect RNase Block (purple cap) | SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C | Thaw on ice | **page 105** |
| SureSelect Fast Hybridization Buffer (bottle) | SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C | Thaw and keep at Room Temperature | **page 105** |
| Probe | –80°C | Thaw on ice | **page 105** |

## Pre-program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 67** for the SureSelect XT HS Human All Exon V8/V8+UTR Probes or in **Table 68** for all other probes. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

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

**Table 67 Hybridization program for *SureSelect XT HS Human All Exon V8/V8+UTR Probes****

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

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

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

‡ Samples are held at 65°C until they are processed in the Capture & Wash automation protocol that begins on **page 112**.

**Table 68 Hybridization program for *all other probes****

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

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

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

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

** Samples are held at 65°C until they are processed in the Capture & Wash automation protocol that begins on **page 112**.

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

**NOTE** The Hybridization reaction may be run overnight with the following protocol modifications.

- In the final segment of the thermal cycler program (**Table 67** or **Table 68**), replace the 65°C Hold step with a 21°C Hold step.
- The hybridized samples may be held at 21°C for up to 16 hours. Begin the capture preparation steps on **page 112** on day 2, after the overnight hold.

## 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 chiller, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Place red PCR plate inserts at Bravo deck positions 4 and 6.
5. Place an empty tip box on shelf 1 of cassette 4 of the workstation MiniHub.
6. Load tip boxes for the run in the BenchCel Microplate Handling Workstation according to **Table 69**.

**Table 69 Initial BenchCel configuration for Hyb_XT_HS2_ILM protocol**

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

## Prepare the Block master mix

1 Prepare the appropriate volume of Block master mix, on ice, as indicated in **Table 70**. Mix by vortexing at medium speed for 15–20 seconds. Keep on ice.

**Table 70 Preparation of Block master mix**

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

## Prepare one or more Probe Hybridization master mixes

1 Prepare the appropriate volume of Probe Hybridization master mix for each of the Probes that will be used for hybridization as indicated in **Table 71** to **Table 74**. Mix by vortexing at medium speed for 15–20 seconds, then spin down briefly. Keep the Probe Hybridization master mix(es) on ice.

**NOTE**

Each row of the prepped cDNA sample plate may be hybridized to a different Probe. However, Probes of different sizes require different post-capture amplification cycles. Plan experiments such that similar-sized Probes are hybridized on the same plate.

For runs that use a single Probe for all rows of the plate, prepare the master mix as described in Step a (**Table 71** or **Table 72**) on **page 105**.

For runs that use different Probes for individual rows, prepare each master mix as described in Step b (**Table 73** or **Table 74**) on **page 106**.

**a** For runs that use a single Probe for all rows, prepare a master mix as described in **Table 71** or **Table 72**, according to the probe design size.

**Table 71 Preparation of Probe Hybridization master mix for Probes <3 Mb, 8 rows of wells**

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

**Table 72 Preparation of Probe Hybridization master mix for Probes ≥3 Mb, 8 rows of wells**

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

**b** For runs that use different Probes in individual rows, prepare a master mix for each Probe as listed in **Table 73** or **Table 74**, according to the probe design size. The volumes listed in **Table 73** and **Table 74** are for a single row of sample wells. If a given Probe will be hybridized in multiple rows, multiply each of the values below by the number of rows assigned to that Probe.

**Table 73 Preparation of Probe Hybridization master mix for Probes <3 Mb, single row of wells**

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

**Table 74 Preparation of Probe Hybridization master mix for Probes ≥3 Mb, single row of wells**

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

## Prepare the master mix source plate

1 Using an **Armadillo** master mix source plate, prepare the hybridization master mix source plate at room temperature, containing the master mixes prepared in **step 1** and **step 1**. Add the volumes indicated in **Table 75** of each master mix to each well of the indicated column of the plate. When using multiple Probes in a run, add the Probe Hybridization master mix for each probe to the appropriate row(s) of the Armadillo plate. The final configuration of the master mix source plate is shown in **Figure 15**.

**Table 75 Preparation of the master mix source plate for Hyb_XT_HS2_ILM protocol**

| Master Mix Solution | Position on Source Plate | Volume of master mix added per Well of Armadillo Source Plate | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1-Column Runs | 2-Column Runs | 3-Column Runs | 4-Column Runs | 6-Column Runs | 12-Column Runs |
| Block master mix | Column 1 (A1-H1) | 11.0 µL | 19.0 µL | 27.0 µL | 34.9 µL | 50.9 µL | 102.7 µL |
| Probe Hybridization master mix | Column 2 (A2-H2) | 23.3 µL | 38.8 µL | 54.3 µL | 69.8 µL | 108.5µL | 217.0 µL* |

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

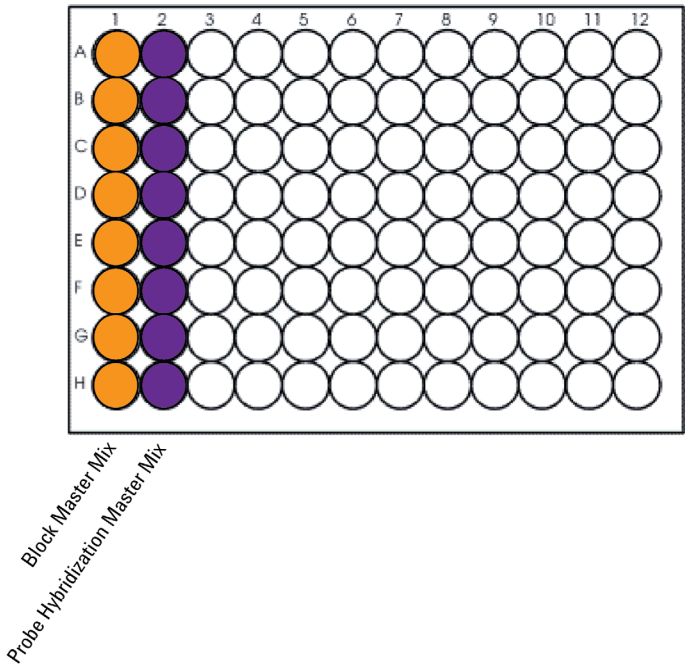



**Figure 15** Configuration of the **Armadillo** master mix source plate for protocol Hyb_XT_HS2_ILM. Column 2 can contain different Probe Hybridization master mixes in each row.

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

## Load the Bravo deck

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

**Table 76 Initial Bravo deck configuration for Hyb_XT_HS2_ILM protocol**

| Location | Content |
|---|---|
| 4 | Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2) |
| 5 | Empty Armadillo plate |
| 6 | Armadillo source plate containing Block master mix and Probe Hybridization master mix (unsealed) seated in red insert |
| 8 | Empty tip box |
| 9 | Prepared library aliquots or library pools in Armadillo plate (unsealed) |

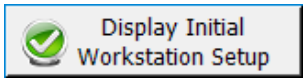
## Run VWorks protocol Hyb_XT_HS2_ILM

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

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

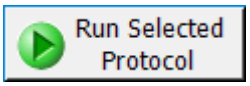
3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

4 Click **Display Initial Workstation Setup**.



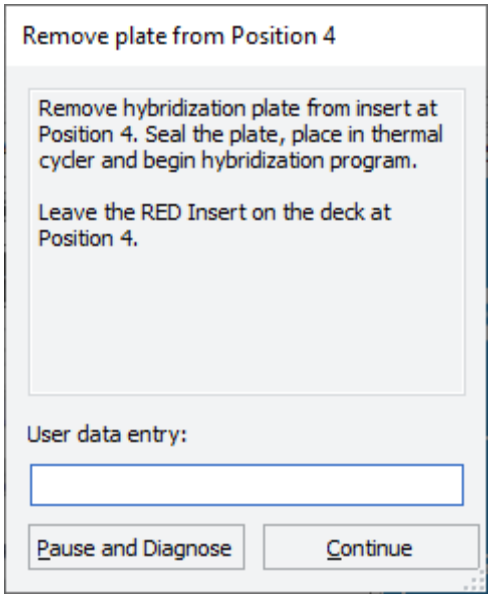

5 Verify that the NGS workstation has been set up as displayed on the right side of the form.

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




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

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




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

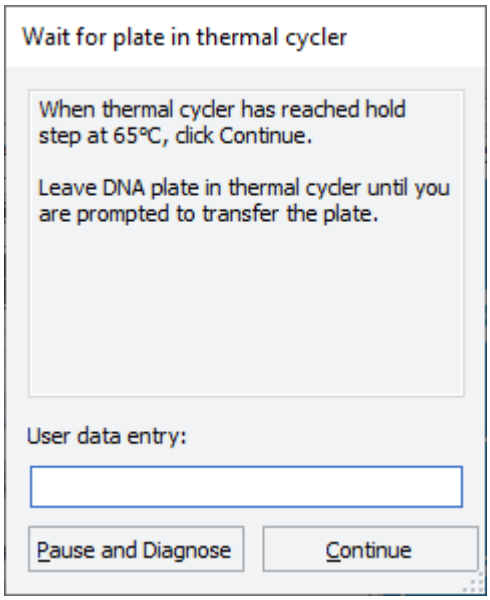
9 Transfer the sealed plate to a thermal cycler. Initiate the pre-programmed thermal cycler program (**Table 67** on page 102 or **Table 68** on page 102).

While the sample plate incubates on the thermal cycler, the Agilent NGS Workstation aliquots the Probe Hybridization master mix to the Armadillo plate.

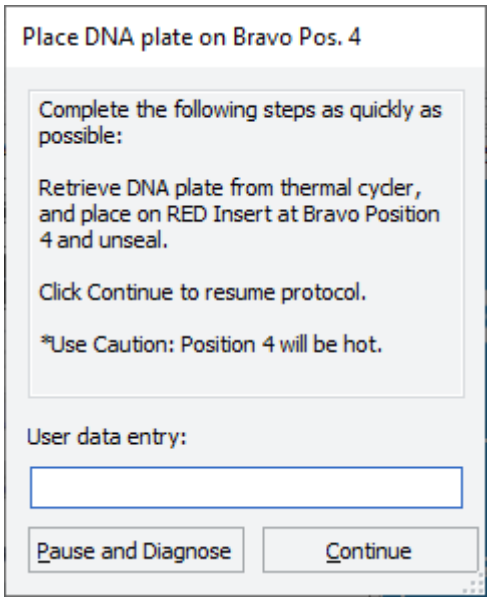
**CAUTION**

You must complete **step 10** to **step 14** quickly, and immediately after being prompted by the VWorks software. It is important that sample temperature remains approximately 65°C during transfers between the Agilent NGS Workstation and thermal cycler.

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




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

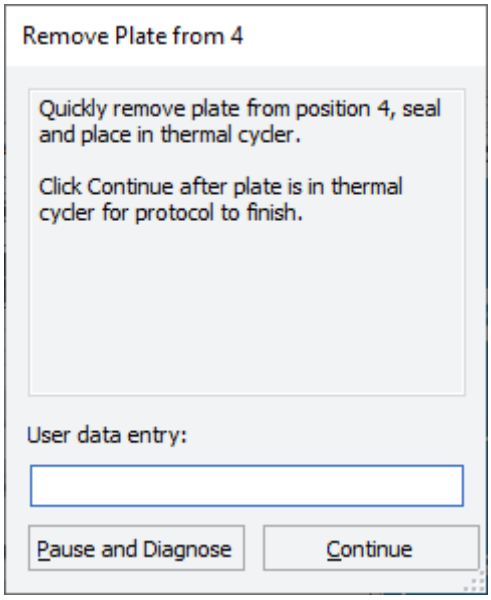



**WARNING** **Bravo deck position 4 will be hot.**

**Use caution when handling components that contact heated deck positions.**

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

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




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

14 Quickly transfer the plate back to the thermal cycler, held at 65°C. On the thermal cycler, initiate the hybridization segment of the pre-programmed thermal cycler program (segment 4 from **Table 67** on page 102 or **Table 68** on page 102). During this step, the prepared DNA samples or DNA sample pools are hybridized to the Probe.

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

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

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

17 When the Hybridization protocol is complete, remove the Armadillo plate containing the remainder of the prepared libraries or library pools. This plate is located at position 9 of the Bravo deck. Seal the plate and store it at –20°C in the event that the samples require further processing.

# Step 3. Capture the hybridized DNA

This step uses runset SSELCapture&Wash_XT_HS2_ILM to automate capture of the cDNA-probe hybrids using streptavidin-coated magnetic beads. Setup tasks for the Capture & Wash protocol (**step 1**, below, through **step 3** on **page 114**) should be completed during the thermal cycler incubation for hybridization (approximately 1.5-hour duration) started on **page 111**.

The Capture & Wash runset uses the components listed in **Table 77**.

**Table 77 Reagents for Capture**

| Kit Component | Storage Location | Where Used |
|---|---|---|
| SureSelect Binding Buffer | SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT | **page 113** |
| SureSelect Wash Buffer 1 | SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT | **page 113** |
| SureSelect Wash Buffer 2 | SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR), RT | **page 113** |
| Dynabeads MyOne Streptavidin T1 bead suspension or SureSelect Streptavidin Beads | Follow storage recommendations provided by supplier (see **Table 1** on page 11) | **page 113** |

## Prepare the workstation

1. Retain the Eppendorf twin.tec source plate containing the Block master mix and Probe Hybridization master mix located at position 6 of the Bravo deck for later use in the Post-CapPCR_XT_HS2_ILM protocol. 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. Place a red PCR plate insert at Bravo deck position 4.
4. Place the silver Deep Well plate insert on position 6 of the Bravo deck. This insert is required to facilitate heat transfer to the Deep Well source plate wells during the Capture & Wash runset. When loading a source plate on the silver insert, make sure the plate is seated properly to ensure proper heat transfer.

## Prepare the streptavidin beads

1. Vigorously resuspend the streptavidin -coated magnetic beads on a vortex mixer. The beads settle during storage.
2. Wash the magnetic beads.
   a. In a conical vial, combine the components listed in **Table 78**. The volumes below include the required overage.

**Table 78 Magnetic bead washing mixture**

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

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

**c** Put the vial into a magnetic separator device.

**d** Remove and discard the supernatant.

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

**3** Resuspend the beads in SureSelect Binding buffer, according to **Table 79** below.

**Table 79 Preparation of magnetic beads for SSELCapture&Wash_XT_HS2_ILM runset**

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

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

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

## Prepare capture and wash solution source plates

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

**2** Prepare an Armadillo source plate labeled *Wash #1*. For each well to be processed, add 150 µL of SureSelect Wash Buffer 1.

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

## Load the Agilent NGS Workstation

1 Load the Labware MiniHub according to **Table 80**, using the plate orientations shown in **Figure 4** on page 50.

**Table 80 Initial MiniHub configuration for SSELCapture&Wash_XT_HS2_ILM runset**

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

2 Load the Bravo deck according to **Table 81** (position 5 should already be loaded).

**Table 81 Initial Bravo deck configuration for SSELCapture&Wash_XT_HS2_ILM runset**

| Location | Content |
|---|---|
| 1 | Empty waste plate (Agilent 2 mL square well) |
| 4 | Empty red insert |
| 5 | Streptavidin beads in Agilent Deep Well source plate |
| 6 | *Wash #2* Deep Well source plate seated on silver insert |

3 Load the BenchCel Microplate Handling Workstation according to **Table 82**.

**Table 82 Initial BenchCel configuration for SSELCapture&Wash_XT_HS2_ILM runset**

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

## Run VWorks runset SSELCapture&Wash_XT_HS2_ILM

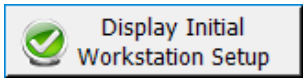
Start the SSELCapture&Wash_XT_HS2_ILM runset upon completion of the hybridization incubation. The hybridization incubation is complete when the thermal cycler program reaches the 65°C Hold step. The 65°C Hold step is segment 6 if using **Table 67** on page 102, and it is segment 5 if using **Table 68** on page 102.

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

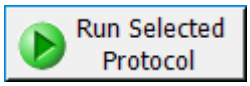
**Table 83 Operator actions during the SSELCapture&Wash_XT_HS2_ILM runset**

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

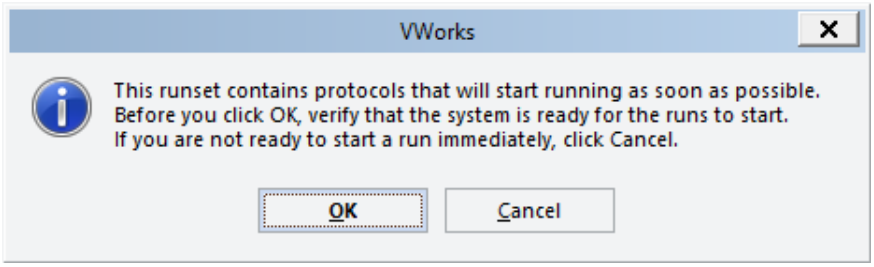
1. On the SureSelect setup form, under **Select protocol to execute**, select the **SSELCapture&Wash_XT_HS2_ILM** runset.
2. Under **Select labware for thermal cycling**, select the specific type of PCR plate used for hybridization. This plate will be transferred from the thermal cycler to Bravo deck position 4 when prompted by VWorks.
3. 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**.

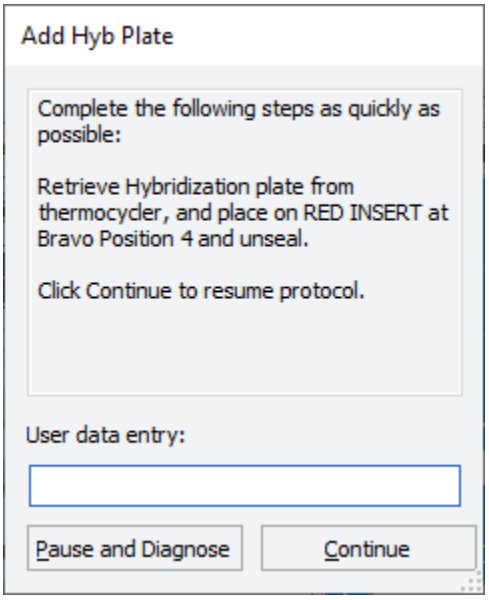



7. When ready to begin the run, click **OK** in the following window.

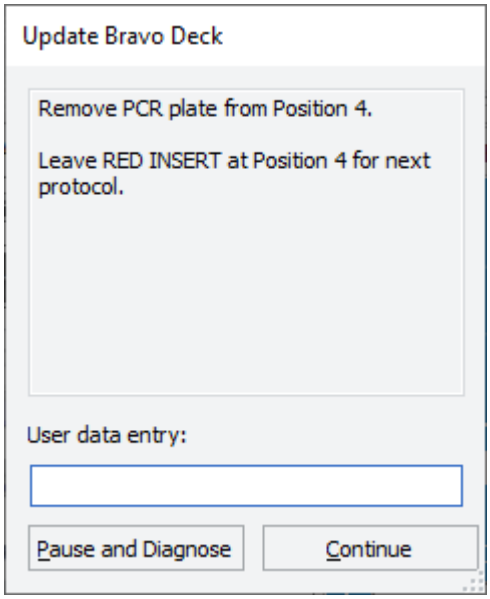



**CAUTION** It is important to complete **step 8** quickly and carefully. Unseal the plate without tilting or jerking the plate to avoid sample splashing. Make sure that the Agilent NGS Workstation is completely prepared, with all components in place, before you transfer the sample plate to the Bravo deck.

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




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




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

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

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

# 7 Post-Capture Sample Processing for Multiplexed Sequencing



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

# Step 1. Amplify the captured libraries

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

The design size of your Probe determines the amplification cycle number. Plan your experiments for amplification of samples prepared using probes of similar design sizes on the same plate. See **Table 86** on page 121 for cycle number recommendations.

This step uses the components listed in **Table 84**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

**Table 84 Reagents for post-capture PCR amplification**

| Component | Storage Location | Mixing Method | Where Used |
|---|---|---|---|
| Herculase II Fusion DNA Polymerase (tube with red cap) | SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C | Pipette up and down 15–20 times | **page 122** |
| 5× Herculase II Reaction Buffer with dNTPs (tube with clear cap) | SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C | Vortexing | **page 122** |
| SureSelect Post-Capture Primer Mix (tube with clear cap) | SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C | Vortexing | **page 122** |

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

## Prepare the workstation

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

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

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

   At the end of the automation protocol, retain this reservoir for use in the AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocol.
5. Pre-set the temperature of Bravo deck positions 4 and 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**.

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

## Pre-program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 85**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

**Table 85 Post-capture PCR Thermal Cycler Program**

| Segment | Number of Cycles | Temperature | Time |
|---|---|---|---|
| 1 | 1 | 98°C | 2 minutes |
| 2 | 12 to 16<br>See **Table 86** for recommendations based on probe design size | 98°C | 30 seconds |
| | | 60°C | 30 seconds |
| | | 72°C | 1 minute |
| 3 | 1 | 72°C | 5 minutes |
| 4 | 1 | 4°C | Hold |

**Table 86 Post-capture PCR cycle number recommendations**

| Probe Size/Description | Cycles |
|---|---|
| Probes <0.2 Mb | 16 cycles |
| Probes 0.2–3 Mb | 14 cycles |
| Probes 3–5 Mb | 13 cycles |
| Probes >5 Mb (including Human All Exon Probes) | 12 cycles |

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

1 Prepare the appropriate volume of post-capture PCR Master Mix, according to **Table 87**. Mix well using a vortex mixer and keep on ice.

**Table 87 Preparation of Post-Capture PCR master mix**

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

2 Using the same **Armadillo** master mix source plate that was used for the Hyb_XT_HS2_ILM protocol run, prepare the master mix source plate by adding the volume of PCR master mix indicated in **Table 88** to all wells of column 3 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 16**

**Table 88 Preparation of the master mix source plate for Post-CapPCR_XT_HS2_ILM protocol**

| Master Mix Solution | Position on Source Plate | Volume of master mix added per well of Eppendorf twin.tec Source Plate | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1-Column Runs | 2-Column Runs | 3-Column Runs | 4-Column Runs | 6-Column Runs | 12-Column Runs |
| Post-Capture PCR Master Mix | Column 3 (A3-H3) | 23.0 µL | 36.0 µL | 49.0 µL | 62.0 µL | 82.0 µL | 163.0 µL |

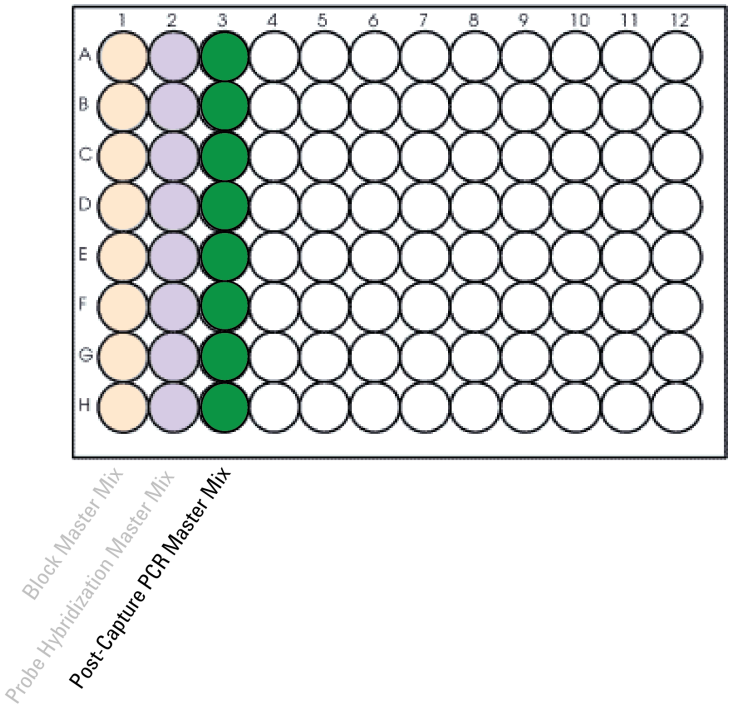



**Figure 16** Configuration of the **Armadillo** master mix source plate for protocol Post-CapPCR_XT_HS2_ILM. The master mixes dispensed during previous protocols are shown in light shading.

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

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

## Load the Agilent NGS Workstation

1 Load the Labware MiniHub according to **Table 89**, using the plate orientations shown in **Figure 4** on page 50.

**Table 89 Initial MiniHub configuration for Post-CapPCR_XT_HS2_ILM protocol**

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

2 Load the Bravo deck according to **Table 90**.

**Table 90 Initial Bravo deck configuration for Post-CapPCR_XT_HS2_ILM protocol**

| Location | Content |
|---|---|
| 4 | Captured DNA bead suspensions in Armadillo plate (unsealed) |
| 6 | Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2) |
| 9 | Armadillo source plate containing Post-Capture PCR master mix (unsealed) |

3 Load the BenchCel Microplate Handling Workstation according to **Table 91**.

**Table 91 Initial BenchCel configuration for Post-CapPCR_XT_HS2_ILM protocol**

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

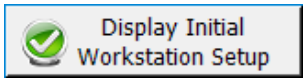
## Run VWorks protocol Post-CapPCR_XT_HS2_ILM

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

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

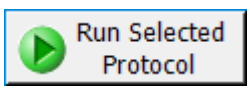
3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

4 Click **Display Initial Workstation Setup**.



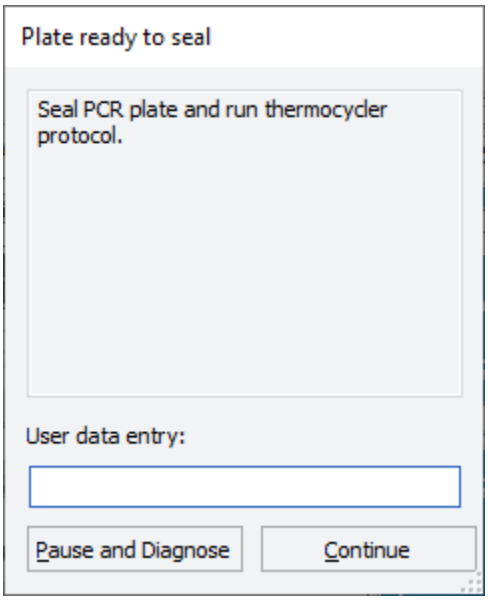

5 Verify that the NGS workstation has been set up as displayed on the right side of the form.

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




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

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




8 Place the plate in the thermal cycler. Resume the thermal cycler program in **Table 85** on page 121.

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

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

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

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

## Prepare the workstation and reagents

1. Retain the Armadillo source plate containing the Post-Capture PCR master mix located at position 9 of the Bravo deck for later use in the TS_HighSensitivity_D1000 protocol (see **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and High Sensitivity D1000 ScreenTape"** on page 129). Otherwise, clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 45°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
5. Prepare an Agilent shallow well reservoir containing 30 mL of nuclease-free water.

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

   Make sure that the water in the reservoir does not contain bubbles. If bubbles are present, dislodge them with a clean pipette tip.
6. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 70% ethanol.
7. Load the Labware MiniHub according to **Table 92**, using the plate orientations shown in **Figure 4** on page 50.

**Table 92 Initial MiniHub configuration for AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocol**

| Vertical Shelf Position | Cassette 1 | Cassette 2 | Cassette 3 | Cassette 4 |
|---|---|---|---|---|
| Shelf 5 (Top) | Aliquotted AMPure XP beads in Agilent Deep Well plate from **page 42** | — | — | — |
| Shelf 4 | — | — | — | — |
| Shelf 3 | — | Empty Armadillo Plate | — | — |
| Shelf 2 | — | Nuclease-free water reservoir from **step 5** | | — |
| Shelf 1 (Bottom) | — | 70% ethanol reservoir from **step 6** | — | Empty tip box |

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

**Table 93 Initial Bravo deck configuration for AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocol**

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

**9** Load the BenchCel Microplate Handling Workstation according to **Table 94**.

**Table 94 Initial BenchCel configuration for AMPureXP_XT_HS2_ILM (Post-Capture PCR) protocol**

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

## Run VWorks protocol AMPureXP_XT_HS2_ILM (Post-Capture PCR)

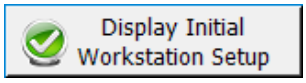
1 On the SureSelect setup form, under **Select protocol to execute**, select the **AMPureXP_XT_HS2_ILM (Post-Capture PCR)** protocol.

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

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

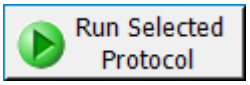
3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

4 Click **Display Initial Workstation Setup**.




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

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




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

# Step 3. Assess sequencing library DNA quantity and quality

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

- Option 1: Prepare the analytical assay plate using automation (protocol TS_HighSensitivity_D1000) and perform analysis on Agilent 4200 TapeStation. See **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and High Sensitivity D1000 ScreenTape"** on page 129.
- 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 134.

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

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

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

### Prepare the 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 **Armadillo** master mix source plate that was used for the Post-CapPCR_XT_HS2_ILM protocol run, prepare the Sample Buffer source plate at room temperature. Add the volume of High Sensitivity D1000 Sample Buffer indicated in **Table 95** to each well of column 4 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 17**.

**Table 95 Preparation of the Sample Buffer Source Plate for TS_HighSensitivity_D1000 protocol**

| Solution | Position on Source Plate | Volume of Sample Buffer added per Well of Armadillo Source Plate | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1-Column Runs | 2-Column Runs | 3-Column Runs | 4-Column Runs | 6-Column Runs | 12-Column Runs |
| High Sensitivity D1000 Sample Buffer | Column 4 (A4-H4) | 8.0 µL | 11.0 µL | 14.0 µL | 17.0 µL | 23.0 µL | 44.0 µL |

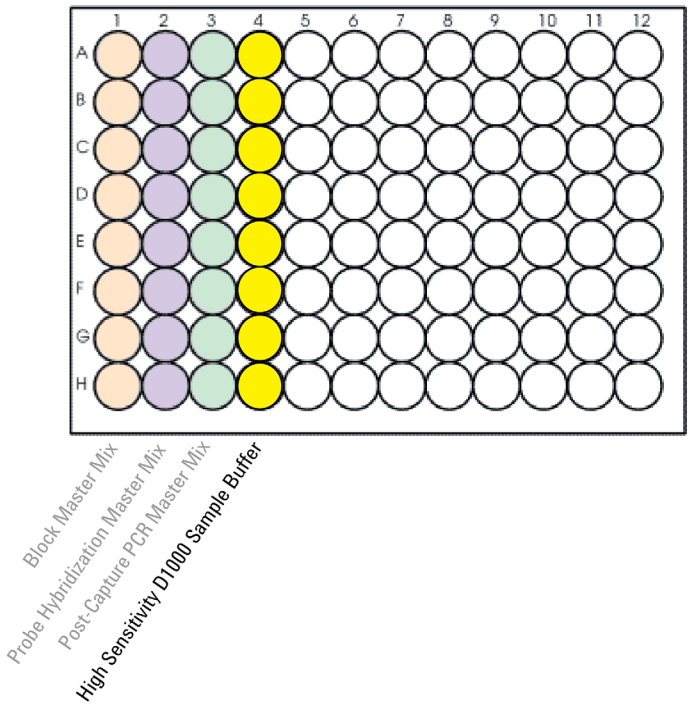



**Figure 17** Configuration of the **Armadillo** source plate for protocol TS_High-Sensitivity_D1000. The master mixes dispensed during previous protocols are shown in light shading.

### Load the Agilent NGS Workstation

**6** Load the Labware MiniHub according to **Table 96**, using the plate orientations shown in **Figure 4** on page 50.

**Table 96 Initial MiniHub configuration for TS_HighSensitivity_D1000 protocol**

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

**7** Load the Bravo deck according to **Table 97**.

**Table 97 Initial Bravo deck configuration for TS_HighSensitivity_D1000 protocol**

| Location | Content |
|---|---|
| 4 | Amplified post-capture libraries or library pools in Armadillo plate (unsealed) |
| 6 | Empty TapeStation analysis plate (Agilent p/n 5042-8502) |
| 9 | Armadillo source plate containing High Sensitivity D1000 Sample Buffer in Column 4 |

**CAUTION** To prevent damage to the Agilent 4200 TapeStation instrument and the Agilent NGS Workstation, use only the specified Agilent plates (Agilent p/n 95042-8502) 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 98**.

**Table 98 Initial BenchCel configuration for TS_HighSensitivity_D1000 protocol**

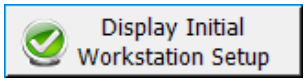
| No. of Columns Processed | Rack 1 | Rack 2 | Rack 3 | Rack 4 |
|---|---|---|---|---|
| 1 | 1 Tip box | — | — | — |
| 2 | 1 Tip box | — | — | — |
| 3 | 1 Tip box | — | — | — |
| 4 | 1 Tip box | — | — | — |
| 6 | 1 Tip box | — | — | — |
| 12 | 1 Tip box | — | — | — |

### Run VWorks protocol TS_HighSensitivity_D1000

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

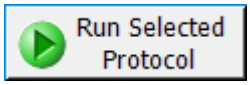
**10** Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

**11** Click **Display Initial Workstation Setup**.



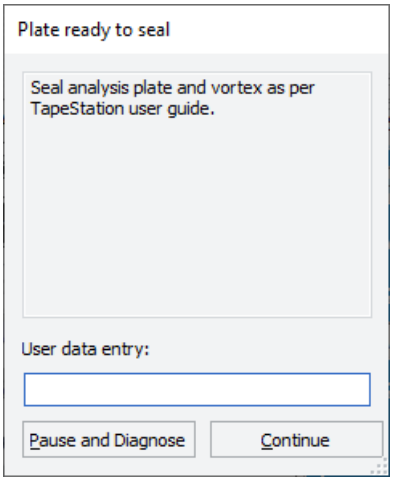

**12** Verify that the NGS workstation has been set up as displayed on the right side of the form.

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




Running the TS_HighSensitivity_D1000 protocol takes approximately 15 minutes. Once removal of analytical samples is complete, remove the primary DNA library sample plate from position 4, seal the plate, and keep on ice until the samples are pooled for sequencing on **page 135**.

**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 assay plate with a foil seal, then vortex and spin the sealed plate as directed in the Agilent High Sensitivity D1000 Assay Quick Guide for 4200 TapeStation System.




**CAUTION**

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

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

### Run the High Sensitivity D1000 Assay and analyze the data

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

**16** Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 99** for guidelines). Sample electropherograms are shown in **Figure 18** (library prepared from high-quality DNA) and **Figure 19** (library prepared from medium-quality FFPE DNA).

**Table 99 Post-capture library qualification guidelines**

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

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

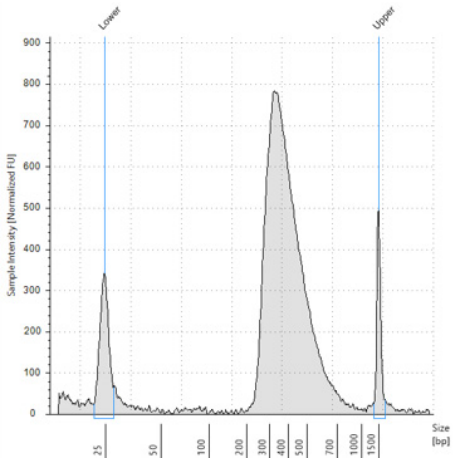



**Figure 18** Post-capture library prepared from an intact RNA sample analyzed using a High Sensitivity D1000 ScreenTape assay

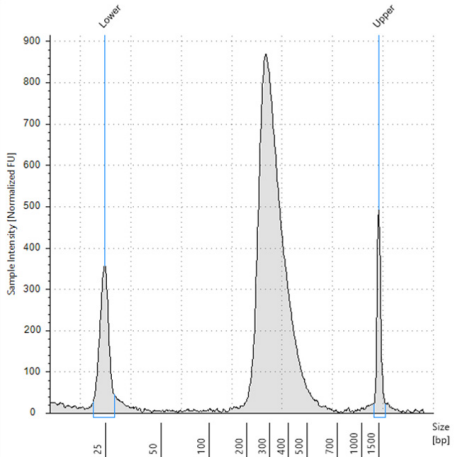



**Figure 19** Post-capture library prepared from a typical FFPE RNA sample analyzed using a High Sensitivity D1000 ScreenTape assay

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

## Option 2: Analysis using an equivalent platform (non-automated)

Using manual preparation of the analytical samples, you can analyze the DNA samples on Agilent 2100 BioAnalyzer, Agilent 5200 Fragment Analyzer, or Agilent 4200/4150 TapeStation. Electropherograms obtained using one of these other analysis platforms are expected to show fragment size profiles similar to those shown for the Agilent 4200 TapeStation (see **Figure 18** through **Figure 19**). Verify that the electropherogram shows the expected DNA fragment size peak position (see **Table 99** for guidelines). **Table 100** includes links to assay instructions.

**Table 100 Post-capture library analysis options**

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

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

# Step 4. Pool samples for multiplexed sequencing (optional)

NOTE

**Pre-Capture Pooling Workflow** If you are using the pre-capture pooling workflow, then your samples were already pooled prior to hybridization with the Probe. The final captured DNA samples contain pools of either 8 or 16 indexed libraries, based on the Probe used and resulting pre-capture pooling strategy. When appropriate for your sequencing platform, the 8-plex or 16-plex samples may be further multiplexed by post-capture pooling. Determine whether to do post-capture pooling by calculating the number of indexes that can be combined per lane, according to the output specifications of the platform used, together with the amount of sequencing data required for your research design. If doing post-capture pooling, use the guidelines provided below. If samples will not be further combined in post-capture pools, proceed to **"Step 5. Prepare sequencing samples"** on page 138.

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

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

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

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

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

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

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

***#*** is the number of indexes, and

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

**Table 101** 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 101 Example of volume calculation for total volume of 20 µL at 10 nM concentration**

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

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

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

1 Create a .csv (comma separated value) file with the headers shown in **Figure 20**. 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 20** shows an example spreadsheet.

- In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
- In the SourceWell field, enter each well position on the source plate containing an amplified and captured indexed library that needs to be added to a pool. Use the Eppendorf twin.tec plate containing the purified indexed libraries as the source plate.
- In the DestinationWell field, enter the well position on the destination plate for the pool.
- In the Volume field, enter the volume (in µL) of each indexed library to be transferred from the source well to the destination well. The volume for each library is calculated from its concentration.
- For all empty wells on the source plate, delete the corresponding rows in the .csv file.

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

**Figure 20** Sample spreadsheets for method 1 and method 2

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\XT_HS2_ILM_v.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_Captures protocol run. If you are using the sample file as a template for runs with fewer than 12 columns, be sure to delete rows for any unused columns on the plate.

3 Load the .csv file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B\XT_HS2_RNA_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.

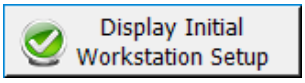
5 Load the Bravo deck according to **Table 102**.

**Table 102 Initial Bravo deck configuration for Aliquot_Captures protocol**

| Location | Content |
|---|---|
| 5 | Empty Agilent Deep Well plate |
| 6 | Empty tip box |
| 8 | New tip box |
| 9 | Purified amplified indexed libraries in Armadillo plate |

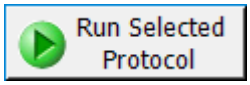
6 On the SureSelect setup form, under **Select protocol to execute**, select the **Aliquot_Captures** protocol.

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 5. Prepare sequencing samples

The final SureSelect XT HS2 library pool is ready for direct sequencing using standard Illumina paired-end primers and chemistry. Each fragment in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform, as shown in **Figure 21**.

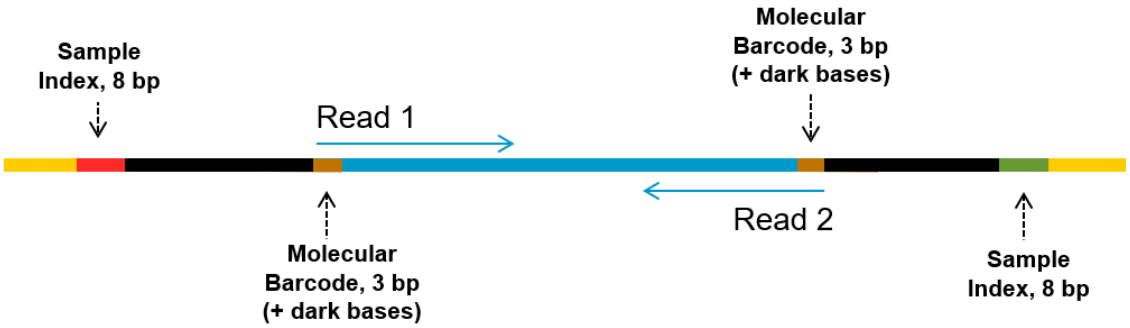



**Figure 21** Content of SureSelect XT HS2 sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the dual sample indexes (red and green), molecular barcodes (brown) and the library PCR primers (yellow).

Libraries can be sequenced on the Illumina HiSeq, MiSeq, NextSeq or NovaSeq platform using the run type and chemistry combinations shown in **Table 103**.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See **Table 103** for kit configurations compatible with the recommended read length.

The optimal seeding concentration for SureSelect XT HS2 target-enriched libraries varies according to sequencing platform, run type, and Illumina kit version. See **Table 103** for guidelines. Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in **Table 103**.

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

**Table 103 Illumina Kit Configuration Selection Guidelines**

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

# Step 6. Do the sequencing run and analyze the data

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

- Each of the sample-level indexes requires an 8-bp index read. For complete index sequence information, see **Table 112** on page 151 through **Table 118** on page 157.
- For the HiSeq, NextSeq, and NovaSeq platforms, set up the run using the instrument's user interface, following the guidelines on **page 140**.
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed on **page 141** to **page 143** to generate a custom sample sheet.
- Do not use the adaptor trimming options in Illumina Experiment Manager (IEM). Make sure any IEM adaptor trimming option checkboxes are cleared (deselected) when setting up the sequencing run. Adaptors are trimmed in later processing steps as described below to ensure proper processing of the degenerate MBCs in the adaptor sequences.
- Demultiplex using Illumina's bcl2fastq, BCL Convert or DRAGEN software to generate paired end reads based on the dual indexes and remove sequences with incorrectly paired P5 and P7 indexes. Do not use the MBC/UMI trimming options in Illumina's demultiplexing software if using Agilent's Genomics NextGen Toolkit (AGeNT) or SureCall software to process your FASTQ files.
- Before aligning reads to the reference genome, Illumina adaptor sequences should be trimmed from the reads using Agilent's AGeNT Trimmer module, which properly accounts for the degenerate MBCs in the adaptor sequence. See **page 144** for more information.
- Library fragments include a degenerate molecular barcode (MBC) in each strand (see **Figure 21** on page 138). 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 144** 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 144**.

## HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface, using the settings shown in **Table 104**. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface and enter the Cycles settings in **Table 104**.

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

**Table 104 Run settings**

| Run Segment | Cycles/Read Length |
|---|---|
| Read 1 | 100 |
| Index 1 (i7) | 8 |
| Index 2 (i5) | 8 |
| Read 2 | 100 |

## 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 112** on page 151 through **Table 118** on page 157 for nucleotide sequences of the SureSelect XT HS2 index pairs.

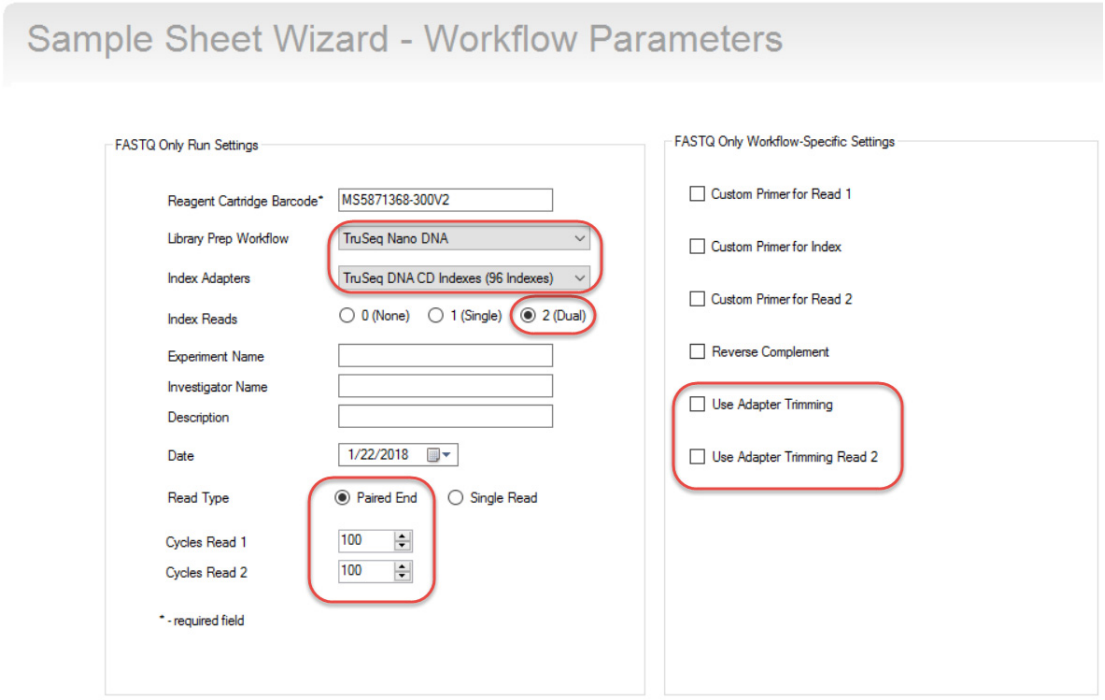
## Set up a custom Sample Sheet:

1 In the IEM software, create a Sample Sheet for the MiSeq platform using the following Workflow selections.

- Under **Category**, select *Other*.
- Under **Application**, select *FASTQ Only*.

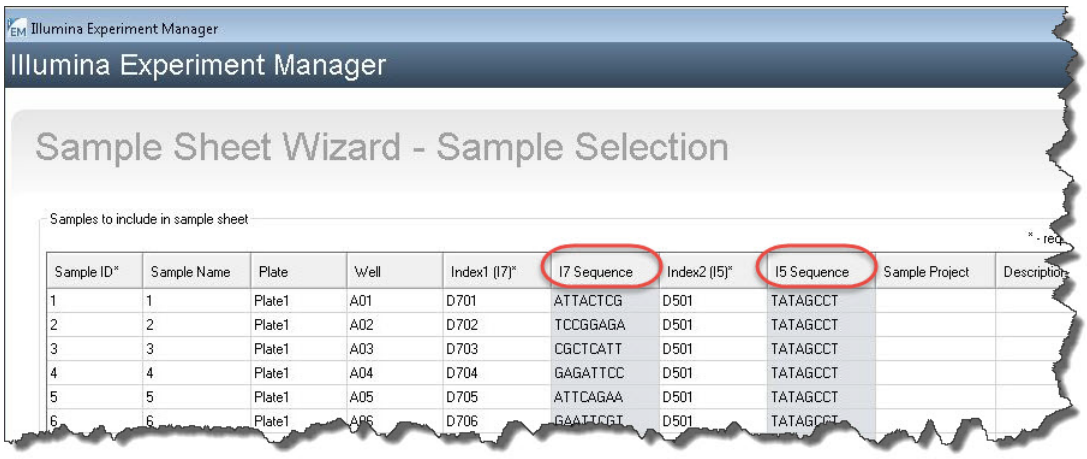
2 On the **Workflow Parameters** screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA.** In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. Clear both adaptor-trimming checkboxes under *FASTQ Only Workflow-Specific Settings*, since adaptor trimming must be performed using Agilent's AGeNT software (see **page 144**).

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




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

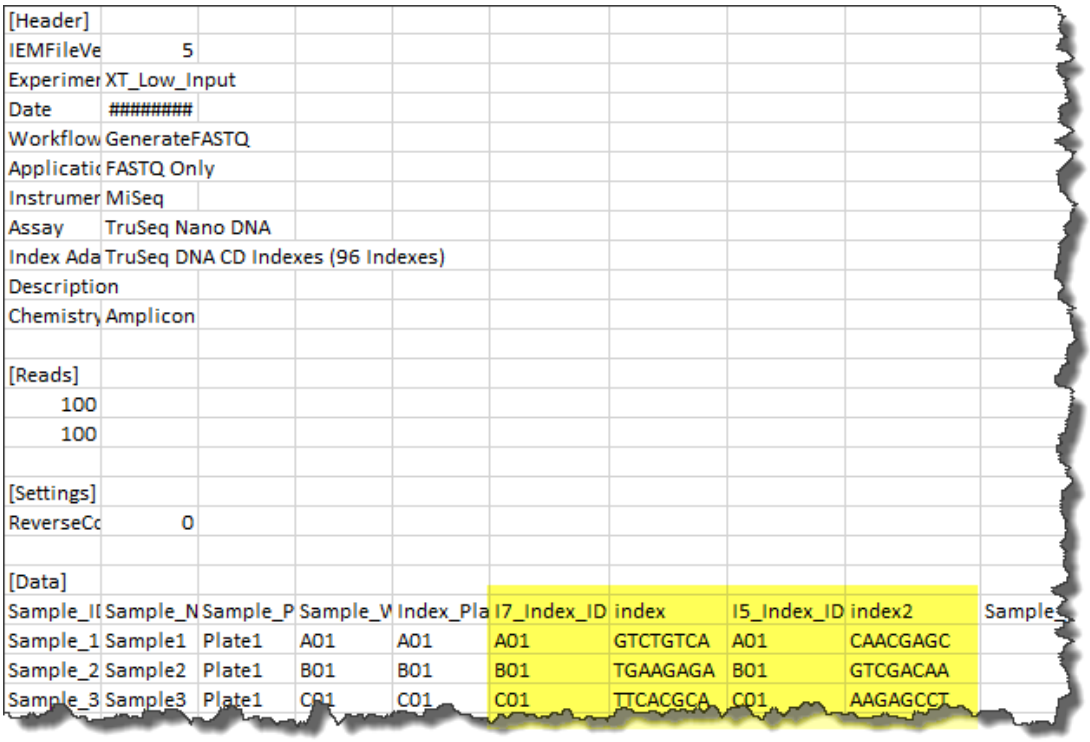
Likewise, in the **I5 Sequence** column, assign any of the Illumina i5 indexes, to be corrected to the i5 sequence from the SureSelect XT HS2 index pair at a later stage.




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

## Editing the Sample Sheet to include SureSelect XT HS2 dual indexes

- Open the Sample Sheet file in a text editor and edit the i7 and i5 index information for each sample in columns 5–8 (highlighted below). See see **Table 112** on page 151 through **Table 118** on page 157 for nucleotide sequences of the SureSelect XT HS2 indexes.
- In column 5 under **I7_Index_ID**, enter the SureSelect XT HS2 index pair number assigned to the sample. In column 6 under **index**, enter the corresponding P7 index sequence.
- In column 7 under **I5_Index_ID**, enter the SureSelect XT HS2 index pair number assigned to the sample. In column 8 under **index2**, enter the corresponding P5 index sequence.
- If the run includes more than 96 samples, the sample sheet may be edited to include additional sample rows containing the assigned SureSelect XT HS2 index pair sequences in column 6 (P7 index) and column 8 (P5 index).



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

**Figure 22** Sample sheet for SureSelect XT HS2 library sequencing

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

# Sequence analysis resources

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

Use Illumina's bcl2fastq, BCL Convert or DRAGEN software to generate paired end reads by demultiplexing sequences based on the dual indexes and to remove sequences with incorrectly paired P5 and P7 indexes.

The demultiplexed FASTQ data needs to be pre- processed to remove sequencing adaptors and extract the molecular barcode (MBC) sequences using the Agilent Genomics NextGen Toolkit (AGeNT). AGeNT is a set of Java- based software modules that provide MBC pre- processing, adaptor trimming and duplicate read identification. This toolkit is designed to enable building, integrating, maintaining, and troubleshooting internal analysis pipelines for users with bioinformatics expertise. For additional information and to download this toolkit, visit the **AGeNT page at www.agilent.com** and review the **AGeNT Best Practices** document for processing steps suitable for XT HS2 RNA libraries.

**NOTE**

If your sequence analysis pipeline excludes MBCs, you can remove the first 5 bases from Read 1 and Read 2 by masking or trimming before proceeding to downstream analysis. If demultiplexing using bcl2fastq, MBCs may be masked by including the base mask **N5Y*,I8,I8,N5Y*** (where * is replaced with the actual read length, matching the read length value in the RunInfo.xml file). If demultiplexing using BCL Convert, MBCs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y*;I8;I8;N5Y*** (where * is replaced with read length after trimming, e.g., use N5Y145;I8;I8;N5Y145 for 2x150 NGS).

Alternatively, the first 5 bases may be trimmed from the demultiplexed fastq files using a suitable processing tool of your choice, such as seqtk. The AGeNT Trimmer module can also be used to remove the MBCs while trimming adaptor sequences. Standard adaptor trimmers will fail to remove the MBC sequences from the opposite adaptor (refer to **Figure 21**), which may affect alignment quality.

The trimmed reads should be aligned using a suitable RNA data alignment tool. Once alignment is complete, the AGeNT CReaK (Consensus Read Kit) tool can be used in the single- strand consensus mode to generate consensus reads and mark or remove duplicates. The resulting BAM files are ready for downstream analysis including gene expression and variant discovery.

**NOTE**

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

## Strandedness guidelines

The SureSelect XT HS2 RNA sequencing library preparation method preserves RNA strandedness using dUTP second- strand marking. The sequence of read 1, which starts at the P5 end, matches the reverse complement of the poly-A RNA transcript strand. Read 2, which starts at the P7 end, matches the poly-A RNA transcript strand. When running analysis of this data to determine strandedness, it is important to include this information. For example, when using the Picard tools (**https://broadinstitute.github.io/picard**) to calculate RNA sequencing metrics, it is important to include the parameter *STRAND_SPECIFICITY=SECOND_READ_TRANSCRIPTION_STRAND* to correctly calculate the strand specificity metrics.

# 8 Reference



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

# Kit Contents

The SureSelect XT HS2 DNA System protocol using the Agilent NGS Workstation uses the kits listed in **Table 105**. Detailed contents of each of the multi-part component kits listed in **Table 105** are shown in **Table 107** through **Table 110** on the following pages.

**Table 105 Component Kits**

| Kit Name (p/n) | Component Kit Name | Component Kit p/n | Storage Condition |
|---|---|---|---|
| SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR), 96 Reactions (G9993A through G9993D) | SureSelect cDNA Module (Pre PCR) | 5500-0149 | –20°C |
| | SureSelect XT HS2 Library Preparation Kit for ILM (Pre PCR) | 5500-0151 | –20°C |
| | SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) | 5191-5688 (Index Pairs 1–96),<br>5191-5689 (Index Pairs 97–192),<br>5191-5690 (Index Pairs 193–288),<br>OR<br>5191-5691 (Index Pairs 289–384) | –20°C |
| SureSelect XT HS2 RNA Target Enrichment Kit, 12 Hybs (G9994A) | SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR) | 5191-6689 | Room Temperature |
| | SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR) | 5191-6690 | –20°C |
| SureSelect XT HS2 RNA Reagent Kit, 96 Reactions (G9991A through G9991D; or G9992A through G9992D with AMPure XP/ Streptavidin Beads) | SureSelect cDNA Module (Pre PCR) | 5500-0149 | –20°C |
| | SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR) | 5500-0151 | –20°C |
| | SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) | 5191-5688 through 5191-5691 | –20°C |
| | SureSelect Target Enrichment Kit, ILM Hyb Module, Box 1 (Post PCR) | 5190-9687 | Room Temperature |
| | SureSelect XT HS2 Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR) | 5191-6688 | –20°C |
| | SureSelect RNA AMPure® XP Beads (included with kits G9992A through G9992D) | 5191-6671 | +4°C |
| | SureSelect Streptavidin Beads (included with kits G9992A through G9992D) | 5191-5742 | +4°C |

**Table 106 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 107 SureSelect XT HS2 RNA Library Preparation Kit for ILM (Pre PCR) Content**

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

**Table 108 SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) Content**

| Kit Component | Format |
|---|---|
| SureSelect XT HS2 Index Primer Pairs for ILM (Pre PCR) | Orange 96-well plate (index pairs 1–96), OR<br>Blue 96-well plate (index pairs 97–192), OR<br>Green 96-well plate (index pairs 193–288), OR<br>Red 96-well plate (index pairs 289–384) |

**Table 109 SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 1 (Post PCR) Content**

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

**Table 110 SureSelect XT HS2 Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR) Content**

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

# SureSelect XT HS2 Index Primer Pair Information

The SureSelect XT HS2 Index Primer Pairs are provided pre-combined. Each member of the primer pair contains a unique 8-bp P7 or P5 index, resulting in dual-indexed NGS libraries. One primer pair is provided in each well of 96-well plates (see **page 159** through **page 160** for plate maps). Each well contains a single- use aliquot of a specific pair of P7 plus P5 primers.

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

The nucleotide sequence of the index portion of each primer is provided in **Table 112** through **Table 118**. 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 111**. Correct representation of the P5 index orientation in sample sheets or during sequencing run setup is crucial to successful demultiplexing. Refer to Illumina support documentation and resources to determine the correct P5 index strand orientation for your application.

**Table 111 P5 index sequencing orientation by Illumina platform**

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

* Some run setup and management tools used with these platforms automatically create the reverse complement sequence for the P5 index sequence entered for the run. Be sure to consult Illumina's support documentation for the combination of platform and tools used in your pipeline to determine the correct index orientation to enter during run setup.

**Table 112 SureSelect XT HS2 Index Primer Pairs 1–48, provided in orange 96-well plate**

| Primer Pair # | Well | P7 Index Forward | P5 Index Forward | P5 Index Reverse Complement | Primer Pair # | Well | P7 Index Forward | P5 Index Forward | P5 Index Reverse Complement |
|---|---|---|---|---|---|---|---|---|---|
| 1 | A01 | CAAGGTGA | ATGGTTAG | CTAACCAT | 25 | A04 | AGATGGAT | TGGCACCA | TGGTGCCA |
| 2 | B01 | TAGACCAA | CAAGGTGA | TCACCTTG | 26 | B04 | GAATTGTG | AGATGGAT | ATCCATCT |
| 3 | C01 | AGTCGCGA | TAGACCAA | TTGGTCTA | 27 | C04 | GAGCACTG | GAATTGTG | CACAATTC |
| 4 | D01 | CGGTAGAG | AGTCGCGA | TCGCGACT | 28 | D04 | GTTGCGGA | GAGCACTG | CAGTGCTC |
| 5 | E01 | TCAGCATC | AAGGAGCG | CGCTCCTT | 29 | E04 | AATGGAAC | GTTGCGGA | TCCGCAAC |
| 6 | F01 | AGAAGCAA | TCAGCATC | GATGCTGA | 30 | F04 | TCAGAGGT | AATGGAAC | GTTCCATT |
| 7 | G01 | GCAGGTTC | AGAAGCAA | TTGCTTCT | 31 | G04 | GCAACAAT | TCAGAGGT | ACCTCTGA |
| 8 | H01 | AAGTGTCT | GCAGGTTC | GAACCTGC | 32 | H04 | GTCGATCG | GCAACAAT | ATTGTTGC |
| 9 | A02 | CTACCGAA | AAGTGTCT | AGACACTT | 33 | A05 | ATGGTAGC | GTCGATCG | CGATCGAC |
| 10 | B02 | TAGAGCTC | CTACCGAA | TTCGGTAG | 34 | B05 | CGCCAATT | ATGGTAGC | GCTACCAT |
| 11 | C02 | ATGTCAAG | TAGAGCTC | GAGCTCTA | 35 | C05 | GACAATTG | CGCCAATT | AATTGGCG |
| 12 | D02 | GCATCATA | ATGTCAAG | CTTGACAT | 36 | D05 | ATATTCCG | GACAATTG | CAATTGTC |
| 13 | E02 | GACTTGAC | GCATCATA | TATGATGC | 37 | E05 | TCTACCTC | ATATTCCG | CGGAATAT |
| 14 | F02 | CTACAATG | GACTTGAC | GTCAAGTC | 38 | F05 | TCGTCGTG | TCTACCTC | GAGGTAGA |
| 15 | G02 | TCTCAGCA | CTACAATG | CATTGTAG | 39 | G05 | ATGAGAAC | TCGTCGTG | CACGACGA |
| 16 | H02 | AGACACAC | TCTCAGCA | TGCTGAGA | 40 | H05 | GTCCTATA | ATGAGAAC | GTTCTCAT |
| 17 | A03 | CAGGTCTG | AGACACAC | GTGTGTCT | 41 | A06 | AATGACCA | GTCCTATA | TATAGGAC |
| 18 | B03 | AATACGCG | CAGGTCTG | CAGACCTG | 42 | B06 | CAGACGCT | AATGACCA | TGGTCATT |
| 19 | C03 | GCACACAT | AATACGCG | CGCGTATT | 43 | C06 | TCGAACTG | CAGACGCT | AGCGTCTG |
| 20 | D03 | CTTGCATA | GCACACAT | ATGTGTGC | 44 | D06 | CGCTTCCA | TCGAACTG | CAGTTCGA |
| 21 | E03 | ATCCTCTT | CTTGCATA | TATGCAAG | 45 | E06 | TATTCCTG | CGCTTCCA | TGGAAGCG |
| 22 | F03 | GCACCTAA | ATCCTCTT | AAGAGGAT | 46 | F06 | CAAGTTAC | TATTCCTG | CAGGAATA |
| 23 | G03 | TGCTGCTC | GCACCTAA | TTAGGTGC | 47 | G06 | CAGAGCAG | CAAGTTAC | GTAACTTG |
| 24 | H03 | TGGCACCA | TGCTGCTC | GAGCAGCA | 48 | H06 | CGCGCAAT | CAGAGCAG | CTGCTCTG |

**Table 113 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 114 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 115 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 116 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 117 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 118 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 119 SureSelect XT HS2 Index Primer Pairs 337–384, provided in red 96-well plate

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

# Index Primer Pair Plate Maps

**Table 120 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 121 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 122 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 123 Plate map for SureSelect XT HS2 Index Primer Pairs 289-384, provided in red plate**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 289 | 297 | 305 | 313 | 321 | 329 | 337 | 345 | 353 | 361 | 369 | 377 |
| B | 290 | 298 | 306 | 314 | 322 | 330 | 338 | 346 | 354 | 362 | 370 | 378 |
| C | 291 | 299 | 307 | 315 | 323 | 331 | 339 | 347 | 355 | 363 | 371 | 379 |
| D | 292 | 300 | 308 | 316 | 324 | 332 | 340 | 348 | 356 | 364 | 372 | 380 |
| E | 293 | 301 | 309 | 317 | 325 | 333 | 341 | 349 | 357 | 365 | 373 | 381 |
| F | 294 | 302 | 310 | 318 | 326 | 334 | 342 | 350 | 358 | 366 | 374 | 382 |
| G | 295 | 303 | 311 | 319 | 327 | 335 | 343 | 351 | 359 | 367 | 375 | 383 |
| H | 296 | 304 | 312 | 320 | 328 | 336 | 344 | 352 | 360 | 368 | 376 | 384 |

# Quick Reference Tables for Master Mixes and Source Plates

This section contains copies of the tables for master mix formulations and source plate volumes used in the SureSelect XT HS2 RNA System Protocol using Agilent NGS Workstation protocol.

## Fragmentation of intact RNA

**Table 124 Fragmentation Master Mix source plate for protocol Fragmentation_XT_HS2_RNA - used on page 49**

| 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 125 First Strand cDNA master mix source plate for protocol FirstStrandcDNA_XT_HS2_RNA - used on page 55**

| 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) | 14.5 µL | 24.0 µL | 33.5 µL | 43.0 µL | 67.0 µL | 129.0 µL |

**Table 126 Second Strand master mix - used on page 60**

| Reagent | Volume for 1 Library | Volume for 1 Column | Volume for 2 Columns | Volume for 3 Columns | Volume for 4 Columns | Volume for 6 Columns | Volume for 12 Columns |
|---|---|---|---|---|---|---|---|
| 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 127 Second Strand master mix source plate for protocol SecondStrandcDNA_XT_HS2_RNA - used on page 60**

| 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 128 End Repair/dA-Tailing master mix - used on page 67**

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

**Table 129 Ligation master mix - used on 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 | 1270.8 µL | 2737 µL |
| T4 DNA Ligase (tube with blue cap) | 2 µL | 25.5 µL | 42.5 µL | 59.5 µL | 76.5 µL | 110.5 µL | 238 µL |
| **Total Volume** | **25 µL** | **318.8 µL** | **531.3 µL** | **743.8 µL** | **956.3 µL** | **1381.3 µL** | **2975 µL** |

**Table 130 Adaptor Oligo Mix dilution - used on 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 | 143.5 µL | 276.3 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 | 287.0 µL | 552.5 µL |
| **Total Volume** | **7.5 µL** | **127.5 µL** | **191.3 µL** | **255.0 µL** | **318.8 µL** | **430.5 µL** | **828.8 µL** |

**Table 131 Master mix source plate for runset LibraryPrep_XT_LI_ILM - used on page 68**

| 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 | 136.0 µL | 280.0 µL |
| Ligation master mix | Column 5 (A5-H5) | 36.0 µL | 62.0 µL | 88.0 µL | 114.0 µL | 166.0 µL | 360.0 µL |
| Adaptor Oligo Mix dilution | Column 6 (A6-H6) | 15.0 µL | 22.5 µL | 30.0 µL | 37.5 µL | 52.5 µL | 101.3 µL |

## Pre-Capture PCR

**Table 132 Pre-Capture PCR master mix - used on page 74**

| 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 133 Master mix source plate for protocol Pre-CapPCR_XT_LI_ILM - used on page 74**

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

**Table 134 Sample Buffer source plate for TS_D1000 protocol - used on page 81**

| Solution | Position on Source Plate | Volume of Sample Buffer added per Well of Armadillo 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 |

## Hybridization

**Table 135 Block master mix - used on page 104**

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

**Table 136 Probe master mix for Probes <3 Mb, 8 rows of wells - used on page 105**

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

**Table 137 Probe master mix for Probes ≥3 Mb, 8 rows of wells - used on page 105**

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

**Table 138 Probe master mix for Probes <3 Mb, single row of wells - used on page 106**

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

**Table 139 Probe master mix for Probes ≥3 Mb, single row of wells - used on page 106**

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

**Table 140 Master mix source plate for protocol Hyb_XT_LI_ILM - used on page 107**

| **Master Mix Solution** | **Position on Source Plate** | **Volume of master mix added per Well of Armadillo Source Plate** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **1-Column Runs** | **2-Column Runs** | **3-Column Runs** | **4-Column Runs** | **6-Column Runs** | **12-Column Runs** |
| Block master mix | Column 1 (A1-H1) | 11.0 µL | 19.0 µL | 27.0 µL | 34.9 µL | 50.9 µL | 102.7 µL |
| Probe Hybridization master mix | Column 2 (A2-H2) | 23.3 µL | 38.8 µL | 54.3 µL | 69.8 µL | 108.5µL | 217.0 µL* |

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

## Hybrid Capture and Washing

**Table 141 Magnetic bead washing mixture - used on page 113**

| 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 |
|---|---|---|---|---|---|---|---|
| Dynabeads MyOne Streptavidin T1 bead suspension or SureSelect Streptavidin Beads | 50 µL | 425 µL | 825 µL | 1225 µL | 1.65 mL | 2.5 mL | 5.0 mL |
| SureSelect Binding Buffer | 0.2 mL | 1.7 mL | 3.3 mL | 4.9 mL | 6.6 mL | 10 mL | 20 mL |
| **Total Volume** | **0.25 mL** | **2.125 mL** | **4.125 mL** | **6.125 mL** | **8.25 mL** | **12.5 mL** | **25 mL** |

**Table 142 Resuspension of magnetic beads - used on page 113**

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

## Post-Capture PCR

**Table 143 Post-Capture PCR master mix - used on page 122**

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

Table 144 Master mix source plate for post-capture PCR protocol Post-CapPCR_XT_LI_ILM - used on page 122

| Master Mix Solution | Position on Source Plate | Volume of master mix added per well of Armadillo Source Plate | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1-Column Runs | 2-Column Runs | 3-Column Runs | 4-Column Runs | 6-Column Runs | 12-Column Runs |
| Post-Capture PCR Master Mix | Column 3 (A3-H3) | 23.0 µL | 36.0 µL | 49.0 µL | 62.0 µL | 82.0 µL | 163.0 µL |

Table 145 Sample Buffer Source Plate for TS_HighSensitivity_D1000 protocol - used on page 129

| Solution | Position on Source Plate | Volume of Sample Buffer added per Well of Armadillo Source Plate | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1-Column Runs | 2-Column Runs | 3-Column Runs | 4-Column Runs | 6-Column Runs | 12-Column Runs |
| High Sensitivity D1000 Sample Buffer | Column 4 (A4-H4) | 8.0 µL | 11.0 µL | 14.0 µL | 17.0 µL | 23.0 µL | 44.0 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 146 Total RNA Input by Grade**

| RNA grade | DV200 | Recommended input amount | Minimum input amount |
|---|---|---|---|
| Intact RNA (from fresh or frozen samples) | >50% | 200 ng | 10 ng |
| Good FFPE RNA | >50% | 200 ng | 10 ng |
| Poor FFPE RNA | 20% to 50% | 200 ng | 50 ng* |
| Inapplicable FFPE RNA | <20% | Not recommended for further processing | |

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

**Table 147 XT HS2 Index Primer Pairs Volume on Primer Plate**

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

**Table 148 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 |
| AMPureXP_XT_HS2_ILM (Pre-Cap PCR - SinglePlex) | 50 µL |
| AMPureXP_XT_HS2_ILM (Pre-Cap PCR - MultiPlex) | 50 µL |
| AMPureXP_XT_HS2_ILM (Concentration of Pool) | 180 µL |
| AMPureXP_XT_HS2_ILM (Post-Capture PCR ) | 50 µL |

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

**Table 149 Water and Ethanol Volumes for AMPure XP Protocols**

| Reagent | Volume per Rerservoir |
|---|---|
| 70% ethanol in Agilent deep well reservoir | 50 mL |
| Nuclease-free water in Agilent shallow well reservoir | 30 mL |

# Troubleshooting Guide

### If yield of pre-capture libraries is low

- ✔ The library preparation protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance of the highly viscous buffer and enzyme solutions used in the protocol. Be sure to adhere to all instructions when setting up the reactions.
- ✔ PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the pre-capture PCR cycle number by 1 to 2 cycles.
- ✔ Performance of the solid-phase reversible immobilization (SPRI) purification step may be poor. Verify the expiration date for the vial of AMPure XP beads used for purification. Adhere to all bead storage and handling conditions recommended by the manufacturer. Ensure that the beads are kept at room temperature for at least 30 minutes before use. Use freshly-prepared 70% ethanol for each SPRI procedure.

### If solids observed in the End Repair-A Tailing Buffer

- ✔ Vortex the solution at high speed until the solids are dissolved. The observation of solids when first thawed does not impact performance, but it is important to mix the buffer until all solutes are dissolved.

### If pre-capture library fragment size is different than expected in electropherograms

- ✔ FFPE RNA pre-capture libraries may have a smaller fragment size distribution due to the presence of fragments in the input RNA that are smaller than the target RNA fragment size.
- ✔ 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 pre-capture library electropherograms

- ✔ The presence of a low molecular weight peak, in addition to the expected peak, indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to the samples analyzed on **page 85**. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield of pre-capture libraries.

### If yield of post-capture libraries is low

- ✔ PCR cycle number may require optimization. Repeat library preparation and target enrichment for the sample, increasing the post-capture PCR cycle number by 1 to 2 cycles.
- ✔ The RNA Probe used for hybridization may have been compromised. Verify the expiration date on the Probe vial or Certificate of Analysis. Adhere to the recommended storage and handling conditions. Ensure that the Probe Hybridization Mix is kept on ice until it is dispensed into the master mix source plate, as directed on **page 104**, and that solutions containing the Probe are not held at room temperature for extended periods.

### If post-capture library fragment size is different than expected in electropherograms

✔ DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. When preparing 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 % on-target is observed in library sequencing results

✔ Minimize the amount of time that hybridization reactions are exposed to room temperature conditions during hybridization setup. Locate a thermal cycler in close proximity to the Bravo NGS Workstation to retain the 65°C sample temperature during transfer step (**step 14** on **page 111**).

### If low uniformity of coverage with high AT-dropout is observed in library sequencing results

✔ High AT-dropout may indicate that hybridization conditions are too stringent to obtain the desired level of coverage for AT-rich targets.

- For libraries target-enriched using the SureSelect XT HS Human All Exon V8 Probe or SureSelect XT HS PreCap Human All Exon V8 Probe and the hybridization program in **Table 67** on page 102 (including segment with one-hour incubation at 65°C), repeat target enrichment using the hybridization program in **Table 68** on page 102 (without the one-hour incubation at 65°C segment).
- For all other probes, repeat target enrichment at lower stringency using a modified thermal cycler program for hybridization, reducing the hybridization temperature in segments 4 and 5 from 65°C to 62.5°C or 60°C (see **Table 68** on page 102).

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## In This Book

This guide contains information to run the SureSelect XT HS2 protocol for RNA, with optional pre-capture pooling, using automation protocols provided with the Agilent NGS Workstation Option B.



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