SureSelect^{XT} Low Input Automated Target Enrichment for the Illumina Platform

Automated using Agilent NGS Workstation Option B

Protocol

Version G3, December 2022

SureSelect platform manufactured with Agilent SurePrint technology.

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

Notices

Manual Part Number

G9703-90010

Edition

Version G3, December 2022

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Acknowledgment

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CAUTION

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In this Guide...

This guide provides an optimized protocol for preparation of target-enriched Illumina paired-end multiplexed sequencing libraries using SureSelect^{XT} Low Input 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 Sample Preparation

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

4 Hybridization

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

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

6 Appendix: Using FFPE-derived DNA Samples

This chapter describes the protocol modifications for gDNA isolated from FFPE samples.

7 Reference

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

What's New in Version G3

- Design ID information added to **Table 2** on page 12 for pre-designed SureSelect probes.
- Updates to downstream sequencing support information (see **page 106** to **page 115**). Key updates include guidelines for demultiplexing using Illumina's BCL Convert software (see **page 106**) and support for Agilent's new CReaK tool, replacing the LocatIt tool in AGeNT v3.0 (see **page 114**).
- Support for use of Agilent's Alissa Reporter software for SureSelect XT Low Input DNA library sequence pre-processing and human germline DNA variant analysis (see **page 113**).
- Update to **Notice to Purchaser**.
- New *Note* on cross-platform index equivalence on **page 54**.

What's New in Version G2

- Support for SureSelect XT HS Human All Exon V8+UTR Probe and SureSelect XT HS Human All Exon V8+NCV Probe (see **Table 2** on page 12).
- Updates to the NGS Workstation components user guide part numbers (see **Table 6** on page 18).
- Update of the lower limit for fluid transfer offered by the Bravo platform to 0.3 µL (see **page 18**).

What's New in Version G1

- Updated product part number for SureSelect XT HS Human All Exon V8 Probe (see **Table 2** on page 12).

What's New in Version G0

- Support for SureSelect XT HS Human All Exon V8 Probe (see **Table 2** on page 12).
- Updates to downstream sequencing platform and kit support information (see **Table 85** on page 105).
- New recommendation regarding the use of compression pads with the thermal cycler (see **"Procedural Notes"** on page 10).
- Removal of VWorks software version 13.0.0.1360 from list of supported versions (see **Table 3** on page 13).
- Updated document look and feel.

What's New in Version F0

- Support for automated enzymatic DNA fragmentation using Agilent's SureSelect Enzymatic Fragmentation Kit as alternative to mechanical shearing using the Covaris instrument. To include enzymatic fragmentation in automation workflow, see **"Step 2, Option 1. Prepare fragmented DNA by enzymatic fragmentation"** on page 38 for protocol. To use the Covaris shearing workflow, see **"Step 2, Option 2. Prepare fragmented DNA by mechanical shearing"** on page 46 for protocol. Additionally, see **Table 4** on page 15 for specific materials needed for each option.

- Update to the configuration of the master mix source plate for the LibraryPrep_XT_LI_ILM runset. See **Figure 5** on page 51.
- Support for preparation of dual-indexed sequencing libraries using Agilent's P5 index plate (See **Table 4** on page 15 for materials needed, and for sequencing support see **"Dual-Indexed libraries: HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines"** on page 108 and **"Dual-Indexed libraries: MiSeq platform sequencing run setup guidelines"** on page 111).
- Updates to the mechanical shearing protocol to make use of Covaris 96 microTUBE plates. See **Table 4** on page 15 for materials needed and **"Step 2, Option 2. Prepare fragmented DNA by mechanical shearing"** on page 46 for instructions.
- Updates to the preparation of the pre-capture PCR indexing primer plate and pre-capture PCR master mix so that water is added to the master mix rather than to the indexing primers (see **"Step 4. Amplify adaptor-ligated libraries"** on page 54).
- Update to the footnote in **Table 51** on page 73 regarding guidelines on optimal hybridization temperature.
- Support for updated VWorks runset SSELCapture&Wash_XT_LI_v.B1.2.2.rst. Updates from SSELCapture&Wash_XT_LI_v.B1.1.1.rst include changed temperatures and durations of particular protocol steps.
- Support for VWorks software version 13.1.0.1366 and Agilent NGS Workstation Option B p/n G5574AA (see **Table 3** on page 13, **Table 6** on page 18 and *Note* on **page 21**).
- Updates to Illumina Kit Configuration Selection Guidelines (see **Table 85** on page 105).
- Removal of optical caps from list of optional materials (see **"Optional Materials"** on page 16) and procedural note (**page 10**) regarding use of tube caps.
- Reorganization of the tables in section **"Materials Required"** on page 11 to highlight requirements for specific workflow options.
- Updates to thermal cycler recommendations (see **Table 3** on page 13) and usage instructions.
- Support for 5200 Fragment Analyzer and 4150 TapeStation instruments (see footnote to **"Required Equipment--All Workflows"** on page 13). Also see **page 67** and **page 102** for instructions.
- Addition of **"Quick Reference Tables for Master Mixes and Source Plates** to **Chapter 7**. The tables list the volumes used in all the master mixes and source plates.
- Updates to the recommendations regarding use of the molecular barcodes. See third paragraph on **page 35** and *Note* on **page 106**.

Content

1 Before You Begin

Make sure you have the most current protocol. Go to www.agilent.com and search for G9703-90010.

To prepare libraries for Agilent SureSelect Cancer All-In-One assays, use the protocols detailed in this publication, while implementing the considerations provided in the SureSelect Cancer All-In-One Target Enrichment Product Overview Guide (publication G9702-90100).

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 sample processing for SureSelect^{XT} Low Input Target Enrichment using the Agilent NGS Workstation Option B. For automated sample processing using the Agilent NGS Bravo Option A, see publication G9703-90020, and for non-automated sample processing procedures see publication G9703-90000.

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

Procedural Notes

- Certain protocol steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the Agilent NGS Workstation to allow rapid and efficient plate transfer.
- If your thermal cycler is compatible with the use of compression pads, add a compression pad whenever you load a plate that was sealed with the PlateLoc thermal microplate sealer. The pad improves contact between the plate and the heated lid of the thermal cycler.
- Prepare and load the Agilent NGS Workstation as detailed in each of the protocol steps before initiating each automated protocol run. When loading plates in the workstation's Labware MiniHub, always place plates in the orientation shown in **Figure 4** on page 41.
- 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 workstations 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

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

- DNA sample type: high-quality gDNA derived from fresh samples vs. FFPE-derived gDNA samples
- DNA fragmentation method used in workflow: enzymatic fragmentation vs. mechanical (Covaris-mediated) shearing
- Indexing options: single-indexed (i7) or dual-indexed libraries

To determine the materials required for your unique needs, first see **Table 1** through **Table 3** for the reagents and equipment required for all workflows. See **Table 4** for additional materials needed to complete the protocols using the selected DNA sample type, fragmentation method, and indexing option.

Table 1 Required Reagents--All Sample Types

Description	Vendor and part number
SureSelect XT Low Input Reagent Kit for Illumina (ILM) platforms* 96 reactions†, with Index Primers 1–96 96 reactions†, with Index Primers 97–192‡	Agilent p/n G9703A p/n G9703B
AMPure XP Kit 60 mL 450 mL	Beckman Coulter Genomics p/n A63881 p/n A63882
Dynabeads MyOne Streptavidin T1 10 mL 50 mL	Thermo Fisher Scientific p/n 65602 p/n 65604D
1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276
Qubit BR dsDNA Assay Kit 100 assays 500 assays	Thermo Fisher Scientific p/n Q32850 p/n Q32853
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

* Compatible with HiSeq, MiSeq, NextSeq 500, and NovaSeq 6000 platforms.

† Each 96-reaction kit contains sufficient reagents for 96 reactions in runs that include at least 3 columns of samples per run.

‡ If you intend to prepare dual-indexed libraries, note that Index Primers 97–192 are not optimized for use with **SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM**.

Table 2 Compatible Probes*

Probe Capture Library	Design ID	Part Number/Ordering Information (96 Reactions Automation)
Pre-designed Probes		
SureSelect XT HS Human All Exon V8	S33266340	5191-6875
SureSelect XT HS Human All Exon V8+UTR	S33613271	5191-7403
SureSelect XT HS Human All Exon V8+NCV	S33699751	5191-7409
SSel XT Low Input Human All Exon V7	S31285117	5191-4030
SureSelect XT Human All Exon V6	S07604514	5190-8865
SureSelect XT Human All Exon V6 + UTRs	S07604624	5190-8883
SureSelect XT Clinical Research Exome V2	S30409818	5190-9493
ClearSeq Comprehensive Cancer XT	0425761	5190-8013
Custom Probes†		
SureSelect Custom Tier1 1–499 kb		Please visit the **SureDesign website** to design Custom SureSelect probes and obtain ordering information. Contact the SureSelect support team (see **page 2**) or your local representative if you need assistance. Custom probes are also available in a 480 Reaction package size.
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 customized with additional *Plus* custom content		
SSel XT HS and XT Low Input Human All Exon V7 Plus 1		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.
SSel XT HS and XT Low Input Human All Exon V7 Plus 2		
SureSelect XT Human All Exon V6 Plus 1		
SureSelect XT Human All Exon V6 Plus 2		
SureSelect XT Clinical Research Exome V2 Plus 1		
SureSelect XT Clinical Research Exome V2 Plus 2		

* Protocols in this document are also compatible with bundled SureSelect XT Low Input Reagent Kits + Probes, ordered using p/n G9507A-S and G9508A-S. See **page 124** for more information.

† 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 categories use the same optimized target enrichment protocols detailed in this publication.

Table 3 Required Equipment--All Workflows

Description	Vendor and Part Number
Agilent NGS Workstation Option B Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5522A (VWorks software version 13.1.0.1366 or 11.3.0.1195) OR Agilent p/n G5574AA (VWorks software version 13.1.0.1366)
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Thermal cycler and accessories (including compression pads, if compatible)	Various suppliers *Important*: Not all PCR plate types are supported for use in the VWorks automation protocols for the Agilent NGS Workstation. Select a thermal cycler that is compatible with one of the supported PCR plate types. See supported plate types in the listing below.
PCR plates compatible with the Agilent NGS Workstation and associated VWorks automation protocols	Only the following PCR plates are supported: • 96 ABI PCR half-skirted plates (MicroAmp Optical plates), Thermo Fisher Scientific p/n N8010560 • 96 Agilent semi-skirted PCR plate, Agilent p/n 401334 • 96 Eppendorf Twin.tec half-skirted PCR plates, Eppendorf p/n 951020303 • 96 Eppendorf Twin.tec PCR plates (full-skirted), Eppendorf p/n 951020401
Eppendorf twin.tec full-skirted 96-well PCR plates	Eppendorf p/n 951020401 or 951020619
Thermo Scientific Reservoirs	Thermo Scientific p/n 1064156
Nunc DeepWell Plates, sterile, 1.3-mL well volume	Thermo Scientific p/n 260251
Axygen 96 Deep Well Plate, 2 mL, Square Well (waste reservoirs; working volume 2.2 mL)	Axygen p/n P-2ML-SQ-C E & K Scientific p/n EK-2440
Nucleic acid surface decontamination wipes	DNA Away Surface Decontaminant Wipes, Thermo Scientific p/n 7008, or equivalent
Qubit Fluorometer	Thermo Fisher Scientific p/n Q33238
Qubit Assay Tubes	Thermo Fisher Scientific p/n Q32856
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Microcentrifuge	Eppendorf microcentrifuge, model 5417C or equivalent
Plate centrifuge	Labnet International MPS1000 Mini Plate Spinner, p/n C1000 or equivalent
Magnetic separator	DynaMag-50 magnet, Thermo Fisher Scientific p/n 123-02D or equivalent
Vacuum concentrator	Savant SpeedVac, model DNA120, with 96-well plate rotor, model RD2MP, or equivalent
Multichannel pipette	Rainin Pipet-Lite Multi Pipette or equivalent
Single channel pipettes (10-, 20-, 200-, and 1000-µL capacity)	Rainin Pipet-Lite Pipettes or equivalent
Sterile, nuclease-free aerosol barrier pipette tips	general laboratory supplier
Vortex mixer	general laboratory supplier
Ice bucket	general laboratory supplier

Table 3 Required Equipment--All Workflows (continued)

Description	Vendor and Part Number
Powder-free gloves	general laboratory supplier
DNA Analysis Platform and Consumables*	
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
OR	
Agilent 4200 TapeStation†	Agilent p/n G2991AA
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
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
D1000 Sample Buffer‡	Agilent p/n 5067-5602
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585
High Sensitivity D1000 Sample Buffer‡	Agilent p/n 5067-5603

* DNA samples may also be analyzed using the Agilent 5200 Fragment Analyzer, p/n M5310AA, and associated NGS Fragment Kits (e.g., DNF-473-0500 and DNF-474-0500). Follow the assay instructions provided for each NGS Fragment Kit.

† DNA samples may also be analyzed using the Agilent 4150 TapeStation, p/n G2992AA. ScreenTape devices plus their associated reagent kits, and the 8-well strip tubes and caps listed in this table are compatible with both platforms.

‡ The additional TapeStation assay sample buffers, p/n 5067-5602 and 5067-5603, are required only when sample qualification is performed using VWorks automation protocols TS_D1000 and TS_HighSensitivity_D1000.

Table 4 Additional Required Materials based on DNA Sample Type/Fragmentation Method/Indexing Option

Description	Vendor and Part Number
Required for preparation of high-quality DNA samples (not required for FFPE DNA sample preparation)	
High-quality gDNA purification system, for example: QIAamp DNA Mini Kit 50 Samples 250 Samples	 Qiagen p/n 51304 p/n 51306
Required for preparation of FFPE DNA samples (not required for high-quality DNA sample preparation)	
QIAamp DNA FFPE Tissue Kit, 50 Samples	Qiagen p/n 56404
Deparaffinization Solution	Qiagen p/n 19093
FFPE DNA integrity assessment system: Agilent NGS FFPE QC Kit 16 reactions 96 reactions **OR** TapeStation Genomic DNA Analysis Consumables: Genomic DNA ScreenTape Genomic DNA Reagents	 Agilent p/n G9700A p/n G9700B Agilent p/n 5067-5365 p/n 5067-5366
Required for enzymatic fragmentation of DNA samples (not required for workflows with mechanical shearing)	
SureSelect Enzymatic Fragmentation Kit, 96 Reactions Automation	Agilent p/n 5191-6764
Required for mechanical shearing of DNA samples (not required for workflows with enzymatic fragmentation)	
Covaris Sample Preparation System	Covaris model E220
Covaris 96 microTUBE plate	Covaris p/n 520078
Required for dual indexing (not required for preparation of single-indexed libraries)	
SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM Use only with P7 Index Primers 1–96 (supplied with Reagent Kit p/n G9703A); not optimized for use with P7 Index Primers 97–192 (supplied with Reagent Kit p/n G9703B)	5191-4056

Optional Materials

Table 5 Supplier Information for Optional Materials

Description	Vendor and part number
High-quality gDNA purification system, for example:	
QIAamp DNA Mini Kit	Qiagen
50 Samples	p/n 51304
250 Samples	p/n 51306
Tween 20	Sigma-Aldrich p/n P9416-50ML

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} Low Input 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} Low Input 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 (previously G5562-90000)
VWorks Software	G5415-90068 (VWorks versions 13.1.0.1366), or G5415-90063 (VWorks version 11.3.0.1195)
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.

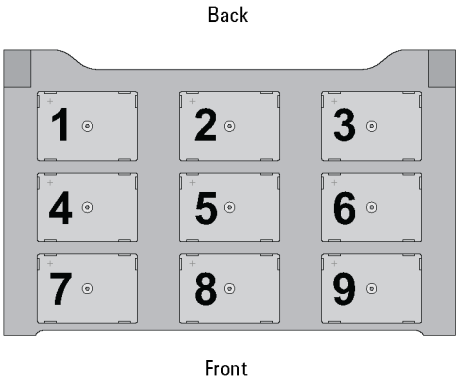


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

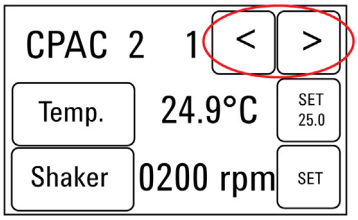
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

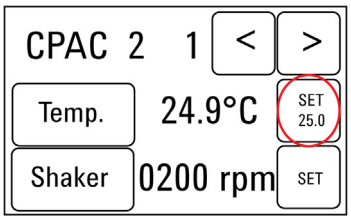
Table 7 Inheco Multi TEC Control touchscreen designations

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

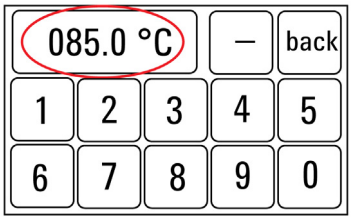
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



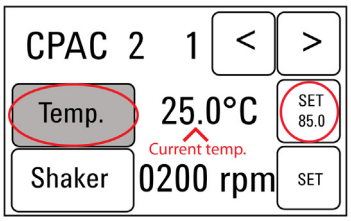
2 To set the temperature of the selected block, press the SET button.



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 or 11.3.0.1195.

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

Logging in to the VWorks software

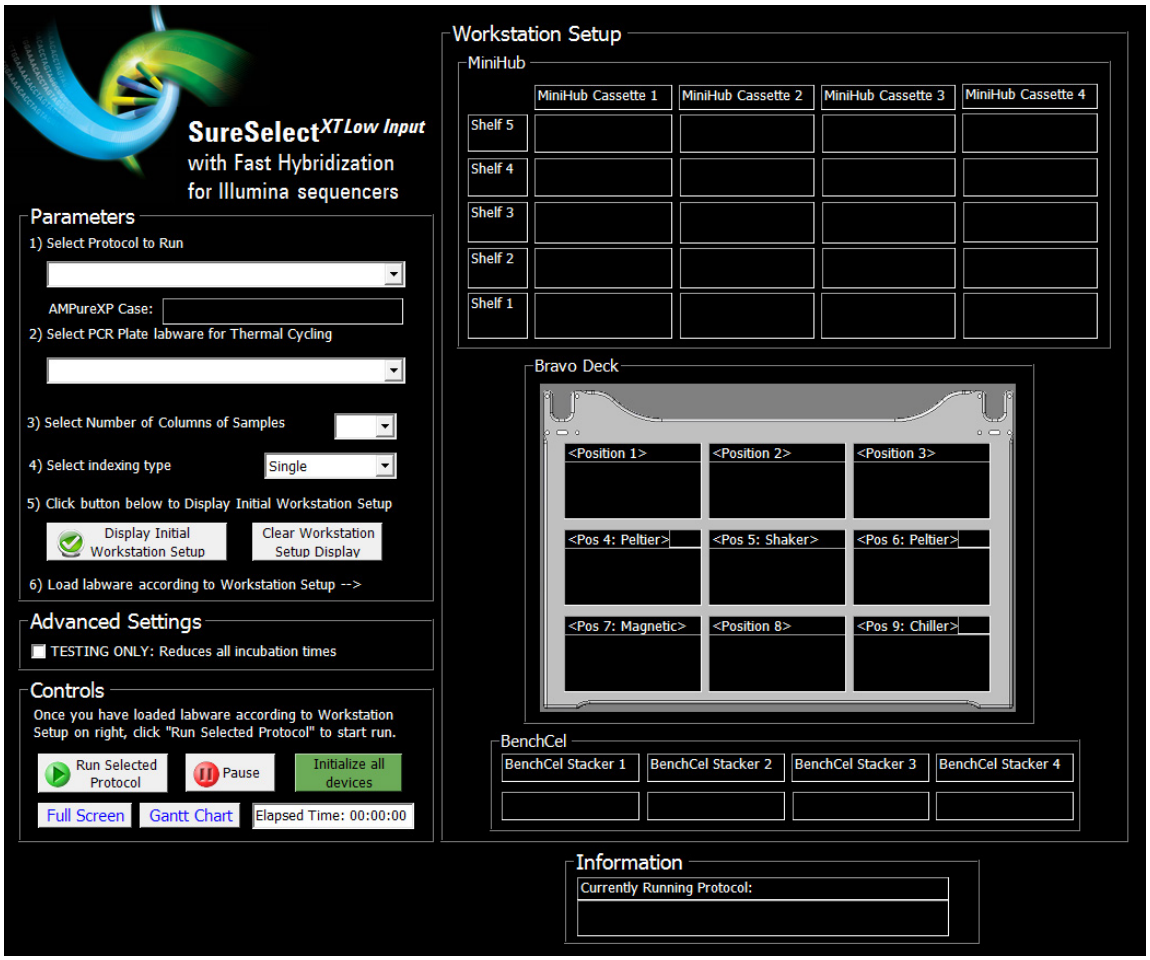
1. Double-click the VWorks icon or the XT Low Input VWorks Form shortcut on the Windows desktop to start the VWorks software.
2. If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
3. In the User Authentication dialog, type your VWorks user name and password, 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} Low Input Form to setup and start a run

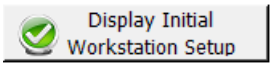
Use the SureSelect^{XT} Low Input VWorks form, shown below, to set up and start each SureSelect automation protocol or runset.



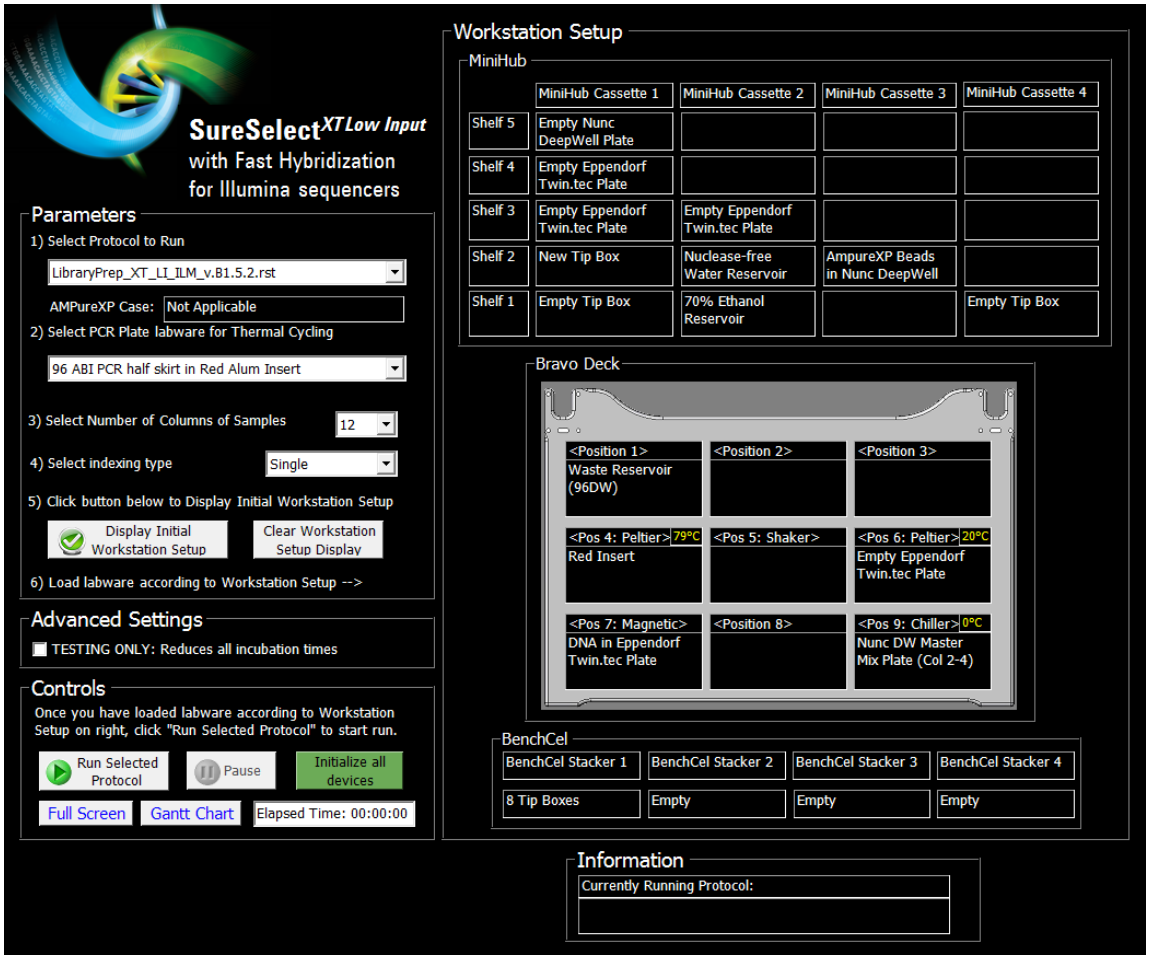
1 Open the form using the shortcut on your desktop.

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 Workstation 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.

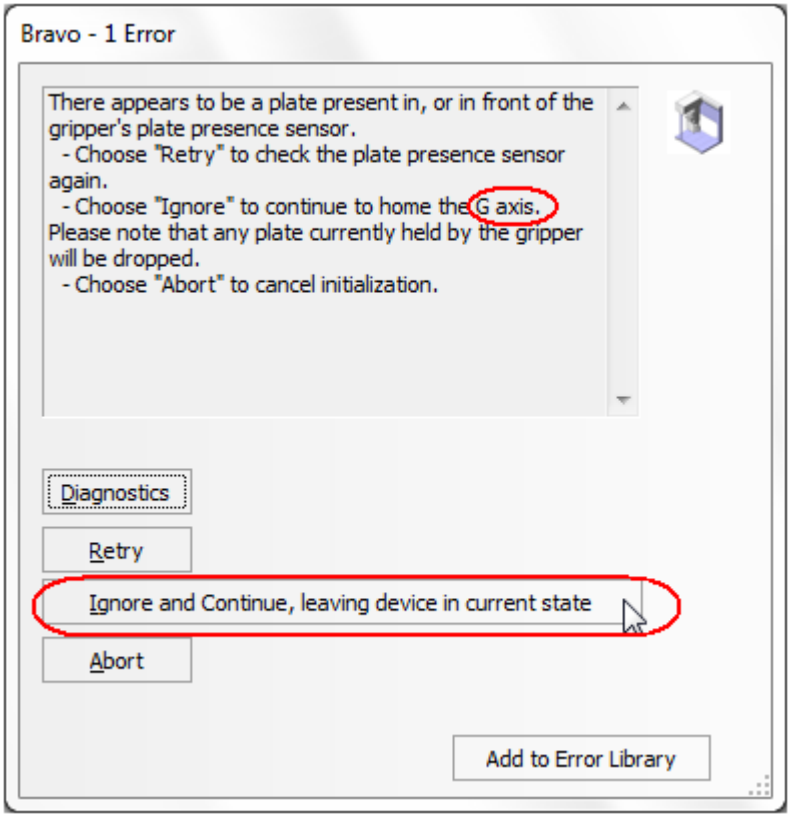


5 After verifying that the NGS Workstation has been set up correctly, click **Run Selected Protocol**.

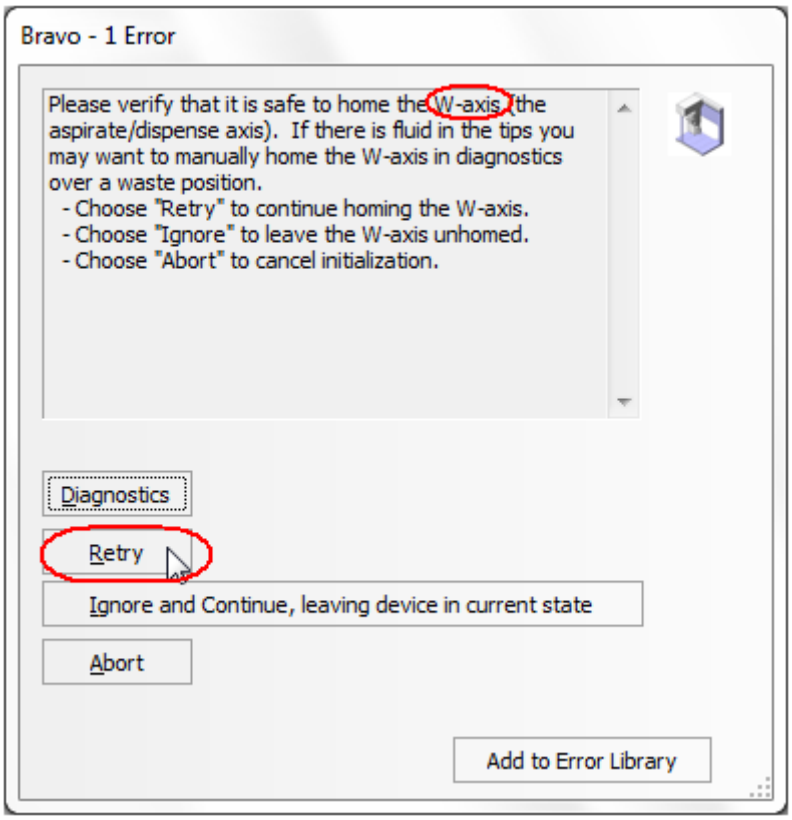
Error messages encountered at start of run

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.

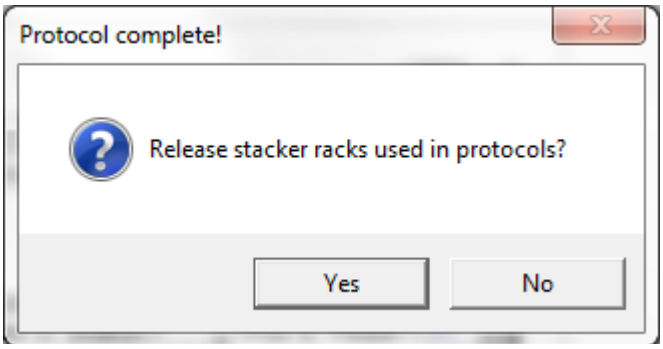
1 Verify that Simulation is off is displayed on the status indicator (accessible by clicking **View > Control Toolbar**).

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_Illumina 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.

Finishing a protocol or runset

The window below appears when each run is complete. Click **Yes** to release the BenchCel racks to allow removal of components used in the current run in preparation for the next .pro or .rst run.



Overview of the SureSelect Target Enrichment Procedure

Figure 2 summarizes the SureSelect^{XT} Low Input target enrichment workflow for 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. Depending on the target size of the SureSelect capture, multiple samples can be pooled and sequenced in a single lane using the 8-bp SureSelect^{XT} Low Input multiplex indexes.

See **Table 9** for a summary of the VWorks protocols used during the workflow. Then, see **Sample 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} Low Input library preparation protocol is compatible with both high-quality gDNA prepared from fresh or fresh frozen samples and lower-quality DNA prepared from FFPE samples, using a DNA input range of 10 to 200 ng DNA.

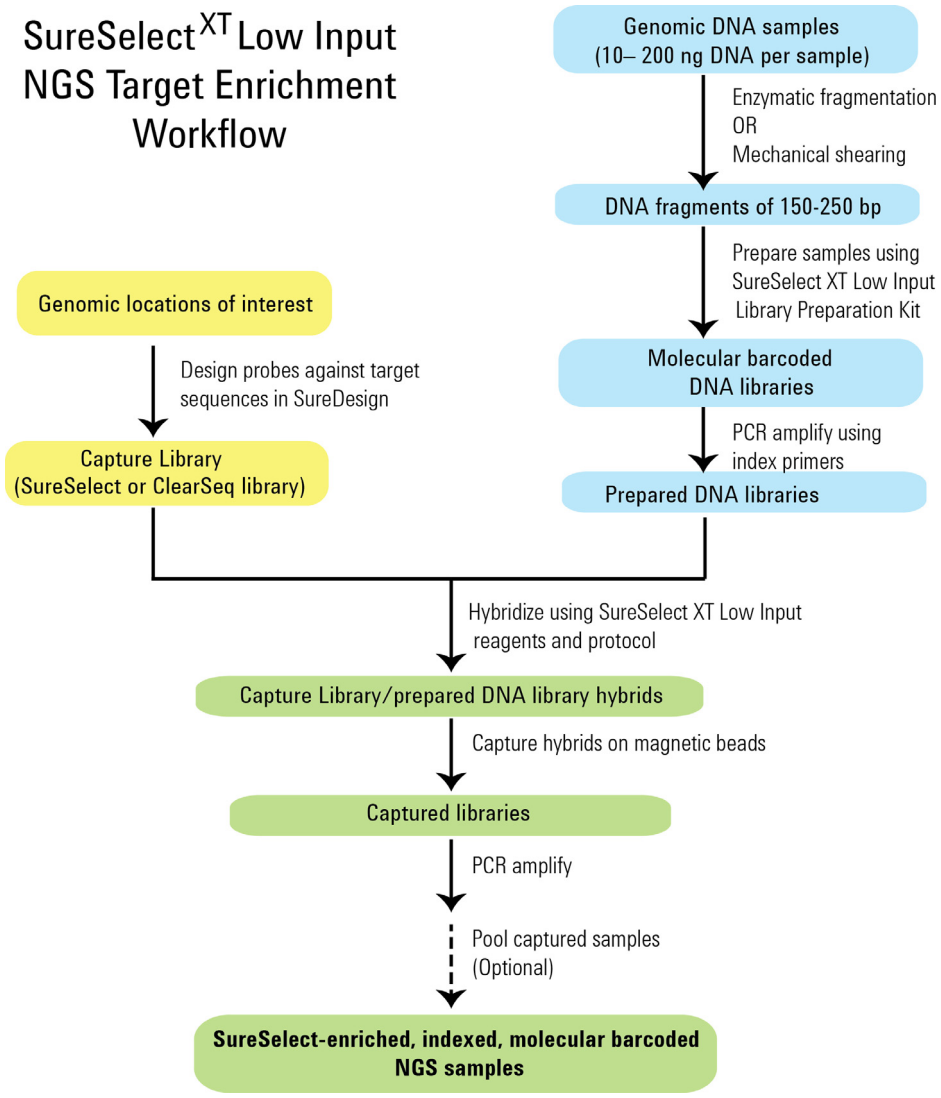


Figure 2 Overall sequencing sample preparation workflow.

Workflow Modulations

The SureSelect^{XT} Low Input target enrichment workflow can be modulated for different applications as described below and summarized in **Table 8** on page 29.

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

DNA Fragmentation Method Automated Enzymatic Fragmentation protocols are provided to support enzymatic fragmentation with automated liquid handling steps (see **"Step 2, Option 1. Prepare fragmented DNA by enzymatic fragmentation"** on page 38). Alternatively, DNA can be mechanically sheared using manual liquid handling steps without automation (see **"Step 2, Option 2. Prepare fragmented DNA by mechanical shearing"** on page 46).

Indexing Options The automated SureSelect^{XT} Low Input target enrichment workflow supports the two different indexing options listed below. Use the *Select indexing type* menu visible on the XT Low Input VWorks Form to indicate your selection.

- Single – Used to generate single index (i7)- and single molecular barcode (i5)-tagged libraries. This strategy uses P7-indexing primers provided in plate format and a single *Adaptor Oligo Mix* reagent solution used for all samples. P5 degenerate molecular barcodes are also supplied for all samples in this solution.
- Dual – Used to generate dual index-tagged libraries. This strategy uses P7-indexing primers provided in plate format and P5-indexed adaptors (without molecular barcodes) also provided in plate format.

Table 8 Summary of workflow modulations supported by the automation protocols

Property	Options	Usage Notes
DNA Sample Integrity	Intact DNA	Use standard protocol with 10-200 ng input DNA
	FFPE DNA	Qualify DNA before use in assay; see **"Protocol modifications for FFPE Samples"** on page 118 for summary of protocol modifications.
DNA Fragmentation Method	Enzymatic Fragmentation	Use the Enzymatic Fragmentation automation protocols to perform enzymatic fragmentation of DNA samples. The protocol EnzFrag_XT_LI_ILM executes the liquid handling steps for the enzymatic fragmentation reactions. The protocol EnzFrag_Dil_XT_LI_ILM directs the dilution of the fragmented samples to the concentration needed for library preparation. Requires purchase of SureSelect Enzymatic Fragmentation Kit, 96 Reactions Automation (Agilent p/n 5191-6764).
	Mechanical (Covaris) Shearing	Perform mechanical shearing on Covaris Sample Preparation System using manual liquid handling (no automated protocol). Requires purchase of Covaris Sample Preparation System and consumables (see **"Additional Required Materials based on DNA Sample Type/Fragmentation Method/Indexing Option"** on page 15).
Indexing Strategy	Single (i7) indexed	Using the *Select indexing type* menu on VWorks interface, select **Single**.
	Dual indexed	Using the *Select indexing type* menu on VWorks interface, select **Dual**. Requires purchase of SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM (Agilent p/n 5191-4056). Designed for use with P7 Indexes 1-96 only.

Automation Protocols used in the Workflow

Table 9 Overview of VWorks protocols and runsets

Workflow Step (Protocol Chapter)	Substep	VWorks Protocols Used for Agilent NGS Workstation automation
Sample Preparation	Shear DNA samples* using enzymatic fragmentation and dilute to appropriate concentration	EnzFrag_XT_LI_ILM EnzFrag_Dil_XT_LI_ILM
	Prepare molecular-barcoded DNA libraries	Runset LibraryPrep_XT_LI_ILM
	Amplify DNA libraries	Pre-CapPCR_XT_LI_ILM
	Purify DNA libraries using AMPure XP beads	AMPureXP_XT_LI_ILM (Case Pre-Capture PCR)
	Analyze amplified libraries using Agilent TapeStation platform	TS_D1000
Hybridization	Aliquot 500-1000 ng of prepped libraries for hybridization	Aliquot_Libraries
	Hybridize prepped DNA (target enrichment)	Hyb_XT_LI_ILM
	Capture and wash DNA hybrids	Runset SSELCapture&Wash_XT_LI
Post-Hybridization	Amplify target-enriched DNA libraries	Post-CapPCR_XT_LI_ILM
	Purify enriched, amplified libraries using AMPure XP beads	AMPureXP_XT_LI_ILM (Case Post-Capture PCR)
	Analyze final libraries using Agilent TapeStation platform	TS_HighSensitivity_D1000

* To shear DNA samples mechanically, rather than enzymatically, perform liquid handing steps manually on the Covaris Sample Preparation System (see **"Step 2, Option 2. Prepare fragmented DNA by mechanical shearing"** on page 46). The XT Low Input VWorks Form does not include an automation protocol for mechanical shearing.

Experimental Setup Considerations for Automated Runs

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

Table 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 gDNA Samples in 96-well Plates for Automated Processing

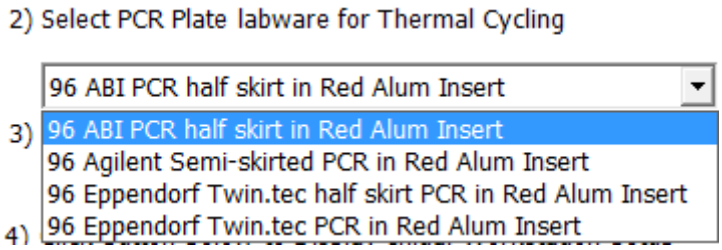
- The Agilent NGS Workstation processes samples column-wise beginning at column 1. gDNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- Samples are P7-indexed during pre-capture amplification (see **Figure 2**). Assign each sample to the appropriate P7-indexing primer during experimental design, and place the sample in the well corresponding to its assigned primer. See **Table 98** and **Table 99** on page 125 for indexing primer plate maps. When preparing dual-indexed libraries, the pre-optimized index pairs are incorporated by using primers from the same well position of the P5 Indexed Adaptors plate and the P7 Indexing Primers 1-96 plate. Importantly, the P5 Indexed Adaptors are not optimized for use with P7 Index Primers 97–192 (supplied with Reagent Kit p/n G9703B).
- At the hybridization step (see **Figure 2**), you can add a different Probe Capture Library 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 70** on page 91 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

3 Sample Preparation

For an overview of the SureSelect^{XT} Low Input target enrichment workflow, see **Figure 2** on page 28. This section contains instructions for automated gDNA library preparation for the Illumina paired-read sequencing platform. For each sample to be sequenced, an individual indexed and molecular-barcoded library is prepared.

The library preparation protocol is compatible with both high-quality gDNA prepared from fresh or fresh frozen samples and lower-quality DNA prepared from FFPE samples. Modifications required for FFPE samples are included throughout the protocol steps. For a summary of modifications for FFPE samples see **Chapter 6** on **page 117**.

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

The library preparation protocol supports the indexing strategies below:

- Single index (i7)- and molecular barcode (i5)-tagged libraries. This strategy adds degenerate molecular-barcoded adaptors to all samples during the adaptor ligation step using the *Adaptor Oligo Mix* reagent.
- Dual index-tagged libraries. This strategy uses P5-indexed adaptors (without molecular barcodes) provided in plate format to add a unique P5 index to each sample during the adaptor ligation step.

Step 1. Prepare and analyze quality of genomic DNA samples

NOTE

If you are preparing DNA samples for an Agilent SureSelect Cancer All-In-One assay, use the following modifications to the gDNA sample preparation instructions in this section:

- Where required for your experimental design, make sure to prepare reference DNA sample(s) alongside your experimental samples
- Use at least 50 ng input gDNA for best results

See publication **G9702-90100** for more information.

Preparation of high-quality gDNA from fresh biological samples

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

NOTE

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

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

Additional qualification of DNA samples is not required for DNA derived from fresh biological samples. Depending on the type of sample fragmentation method to be used, proceed to either **"Step 2, Option 1. Prepare fragmented DNA by enzymatic fragmentation"** on page 38 or **"Step 2, Option 2. Prepare fragmented DNA by mechanical shearing"** on page 46.

Preparation and qualification of gDNA from FFPE samples

1 Prepare gDNA from FFPE tissue sections using Qiagen's QIAamp DNA FFPE Tissue Kit and Qiagen's Deparaffinization Solution, following the manufacturer's protocol. Elute the final gDNA samples from the MinElute column in two rounds, using 30 µL Buffer ATE in each round, for a final elution volume of approximately 60 µL.

NOTE

If tissue lysis appears incomplete after one hour of digestion with Proteinase K, add an additional 10 µL of Proteinase K and continue incubating at 56°C, with periodic mixing, for up to three hours.

Store the gDNA samples on ice for same-day library preparation, or at –20°C for later processing.

2 Assess the quality (DNA integrity) for each FFPE DNA sample using one of the methods below.

Option 1: Qualification using the Agilent NGS FFPE QC Kit (Recommended Method)

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include a ΔΔCq DNA integrity score and the precise quantity of amplifiable DNA in the sample, allowing direct normalization of DNA input for each sample. DNA input recommendations based on ΔΔCq scores for individual samples are summarized in **Table 12**.

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

b Remove a 1 µL aliquot of the FFPE gDNA sample for analysis using the Agilent NGS FFPE QC Kit to determine the ΔΔCq DNA integrity score. See the kit user manual (G9700-90000) at www.agilent.com for more information.

c For all samples with ΔΔCq DNA integrity score ≤1, use the Qubit-based gDNA concentration determined in **step a**, above, to determine volume of input DNA needed for the protocol.

d For all samples with ΔΔCq DNA integrity score >1, use the qPCR-based concentration of amplifiable gDNA, reported by the Agilent NGS FFPE QC Kit results, to determine amounts of input DNA for the protocol.

Table 12 SureSelect XT Low Input DNA input modifications based on ΔΔCq DNA integrity score

Protocol Parameter	non-FFPE Samples	FFPE Samples	
		ΔΔCq≤1*	ΔΔCq >1
DNA input for Library Preparation	10 ng to 200 ng DNA, based on Qubit Assay	10 ng to 200 ng DNA, based on Qubit Assay	10 ng to 200 ng of amplifiable DNA, based on qPCR quantification

* FFPE samples with ΔΔCq scores ≤1 should be treated like non-FFPE samples for DNA input amount determinations. For samples of this type, make sure to use the DNA concentration determined by the Qubit Assay, instead of the concentration determined by qPCR, to calculate the volume required for 10–200 ng DNA.

Option 2: Qualification using Agilent's Genomic DNA ScreenTape assay DIN score

Agilent's Genomic DNA ScreenTape assay, used in conjunction with Agilent's 4200 TapeStation, provides a quantitative electrophoretic assay for DNA sample integrity determination. This assay reports a DNA Integrity Number (DIN) score for each sample which is used to estimate the appropriate normalization of DNA input required for low-integrity DNA samples.

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

b Remove a 1 µL aliquot of the FFPE gDNA sample and analyze using the Genomic DNA ScreenTape assay. See the user manual at www.agilent.com for more information.

c Using the DIN score reported for each sample in the Genomic DNA ScreenTape assay, consult **Table 13** to determine the recommended amount of input DNA for the sample.

Table 13 SureSelect XT Low Input DNA input modifications based on DNA Integrity Number (DIN) score

Protocol Parameter	non-FFPE Samples	FFPE Samples		
		DIN > 8*	DIN 3–8	DIN<3
DNA input for Library Preparation	10 ng to 200 ng DNA, quantified by Qubit Assay	10 ng to 200 ng DNA, quantified by Qubit Assay	Use at least 15 ng for more intact samples and at least 40 ng for less intact samples. Use the maximum amount of DNA available, up to 200 ng, for all samples. Quantify by Qubit Assay.	Use at least 50 ng for more intact samples and at least 100 ng for the least intact samples. Use the maximum amount of DNA available, up to 200 ng, for all samples. Quantify by Qubit Assay.

* FFPE samples with DIN>8 should be treated like non-FFPE samples for DNA input amount determinations.

NOTE DNA quality affects the recommended pre-capture PCR cycle number, as outlined in **Table 35** on page 56. Samples with differing PCR cycle number requirements must be processed in separate library preparation runs.

Step 2, Option 1. Prepare fragmented DNA by enzymatic fragmentation

The SureSelect^{XT} Low Input target enrichment workflow supports two different options for preparing fragmented DNA.

- Option 1, which is described in this section, uses enzymatic fragmentation, with liquid-handling steps executed by automation protocols.
- Option 2 uses mechanical shearing, and is described in **"Step 2, Option 2. Prepare fragmented DNA by mechanical shearing"** on page 46.

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

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

Option 1 uses the components listed in **Table 14**. Thaw and mix each component as directed in **Table 14** before use. Before starting the run, you need to prepare the Fragmentation master mix (with overage) without the DNA sample. **Table 17** provides Fragmentation master mix volumes for runs that include 1, 2, 3, 4, 6, and 12 columns of samples.

Table 14 Reagents thawed before use in protocol

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
5X SureSelect Fragmentation Buffer (blue cap)	SureSelect Enzymatic Fragmentation Kit, –20°C	Thaw on ice then keep on ice	Vortexing	**page 39**
SureSelect Fragmentation Enzyme (green cap)	SureSelect Enzymatic Fragmentation Kit, –20°C	Place on ice just before use	Inversion	**page 39**

Prepare the workstation for protocol EnzFrag_XT_LI_ILM

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Pre-set the temperature of Bravo deck position 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.
5. Place a red PCR plate insert at Bravo deck position 6.

Pre-program the thermal cycler for the fragmentation reaction

6. Pre-program a thermal cycler using the program in **Table 15**. Start the program, then immediately pause the program.

Table 15 Thermal cycler program for enzymatic fragmentation*

Step	Temperature	Time
Step 1	37°C	Varies --see **Table 16**
Step 2	65°C	5 minutes
Step 3	4°C	Hold

* When setting up the thermal cycling program, use a reaction volume setting of 10 µl.

Table 16 Fragmentation duration based on sample type and NGS read length

NGS read length requirement	Target fragment size	Duration of 37°C incubation step	
		High-quality DNA samples	FFPE DNA samples
2 × 100 reads	150 to 200 bp	15 minutes	15 minutes
2 × 150 reads	180 to 250 bp	10 minutes	15 minutes

Prepare the sample plate for fragmentation

7 In wells of an Eppendorf twin.tec plate, dilute 10 ng to 200 ng of each gDNA sample with nuclease-free water to a final volume of 5 µL.

See **Table 12** or **Table 13** for FFPE DNA input guidelines based on the measured DNA quality in each sample.

Prepare the Fragmentation master mix

8 Prepare the appropriate volume of Fragmentation master mix by combining the reagents in **Table 17**.

Mix well by pipetting up and down 20 times or seal the tube and vortex at high speed for 5–10 seconds. Spin briefly to remove any bubbles and keep on ice.

Table 17 Preparation of Fragmentation master mix

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2 µL	34.0 µL	51.0 µL	68.0 µL	85.0 µL	114.8 µL	221.0 µL
5X SureSelect Fragmentation Buffer (blue cap)	2 µL	34.0 µL	51.0 µL	68.0 µL	85.0 µL	114.8 µL	221.0 µL
SureSelect Fragmentation Enzyme (green cap)	1 µL	17.0 µL	25.5 µL	34.0 µL	42.5 µL	57.4 µL	110.5 µL
Total Volume	**5 µL**	**85 µL**	**127.5 µL**	**170 µL**	**212.5 µL**	**287 µL**	**552.5 µL**

Prepare the master mix source plate

9 In an Eppendorf twin.tec plate, prepare the master mix source plate for the run as indicated in **Table 18**. Add the indicated volume of each mixture to all wells of the indicated column of the Eppendorf plate. Keep the master mixes on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in **Figure 3**.

Table 18 Master Mix Source Plate for EnzFrag_XT_LI_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
Fragmentation master mix	Column 1 (A1-H1)	10.0 µL	15.0 µL	20.0 µL	25.0 µL	35.0 µL	68.0 µL

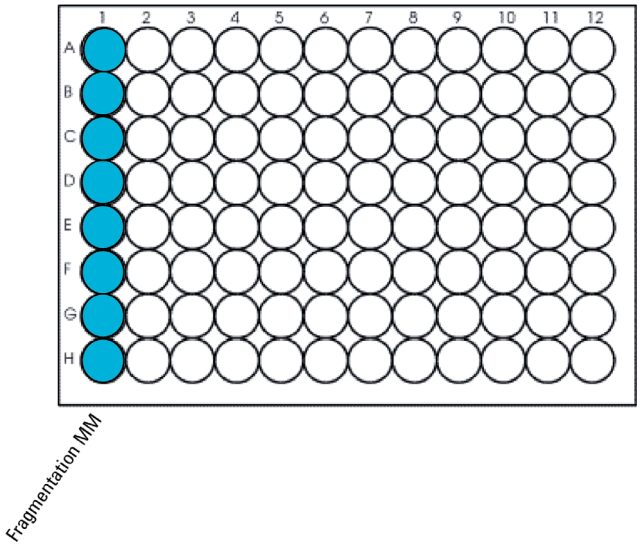


Figure 3 Master mix source plate configuration for EnzFrag_XT_LI_ILM protocol in Eppendorf twin.tec plate

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

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

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

Load the Agilent NGS Workstation

12 Load the Labware MiniHub according to **Table 19**, using the plate orientations shown in **Figure 4**.

Table 19 Initial MiniHub configuration for EnzFrag_XT_LI_ILM protocol

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

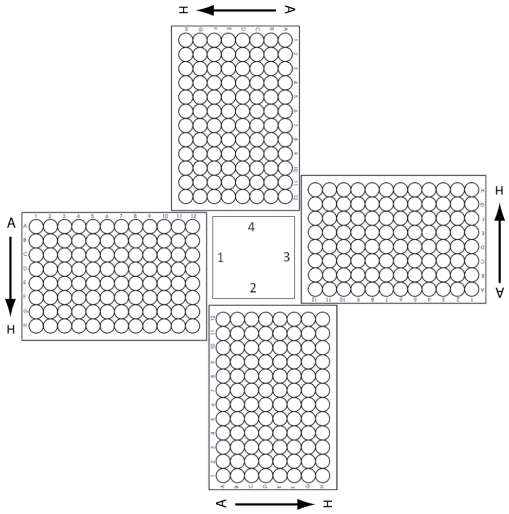


Figure 4 Agilent Labware MiniHub plate orientation. For Thermo Scientific reservoirs, place the notched corner facing the center of the hub.

13 Load the Bravo deck according to **Table 20**.

Table 20 Initial Bravo deck configuration for EnzFrag_XT_LI_ILM protocol

Location	Content
6	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under Parameter 2)
7	gDNA samples in Eppendorf twin.tec plate
9	Fragmentation Master Mix Source Plate, unsealed

14 Load the BenchCel Microplate Handling Workstation according to **Table 21**.

Table 21 Initial BenchCel configuration for EnzFrag_XT_LI_ILM protocol

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

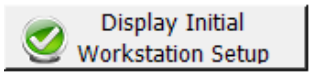
Run VWorks protocol EnzFrag_XT_LI_ILM

15 On the SureSelect setup form, under **Select Protocol to Run**, select the **EnzFrag_XT_LI_ILM** protocol.

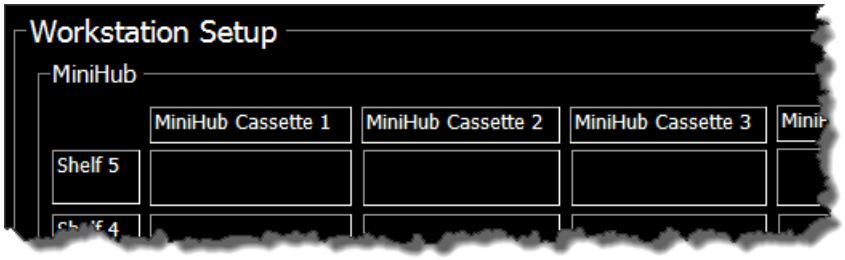
16 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

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

18 Click **Display Initial Workstation Setup**.



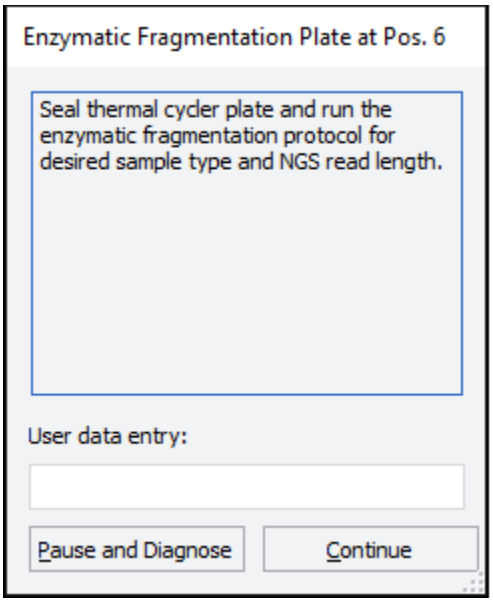
19 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



20 When verification is complete, click R**un Selected Protocol**.

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

21 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 3.0 seconds.



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

23 Immediately place the sample plate in the thermal block. Close the lid and resume the thermal cycling program in **Table 15**.

Prepare the workstation for protocol EnzFrag_Dil_XT_LI_ILM

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

25 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.

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

Prepare the sample dilution water reservoir

27 Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water.

Load the Agilent NGS Workstation

28 Load the Labware MiniHub according to **Table 19**, using the plate orientations shown in **Figure 4**.

Table 22 Initial MiniHub configuration for EnzFrag_Dil_XT_LI_ILM protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 27**	—	—
Shelf 1 (Bottom)	—	—	—	—

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

Table 23 Initial Bravo deck configuration for EnzFrag_Dil_XT_LI_ILM protocol

Location	Content
2	New tip box
5	Empty Eppendorf twin.tec plate (if the PCR plate at position 6 is not an Eppendorf twin.tec PCR plate) OR Empty (if the PCR plate at position 6 is an Eppendorf twin.tec PCR plate)
6	PCR plate containing fragmented DNA samples seated in red insert (PCR plate type must be specified on setup form under Parameter 2)
8	Empty tip box

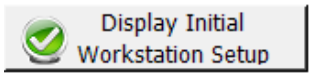
Run VWorks protocol EnzFrag_Dil_XT_LI_ILM

30 On the SureSelect setup form, under **Select Protocol to Run**, select the **EnzFrag_Dil_XT_LI_ILM** protocol.

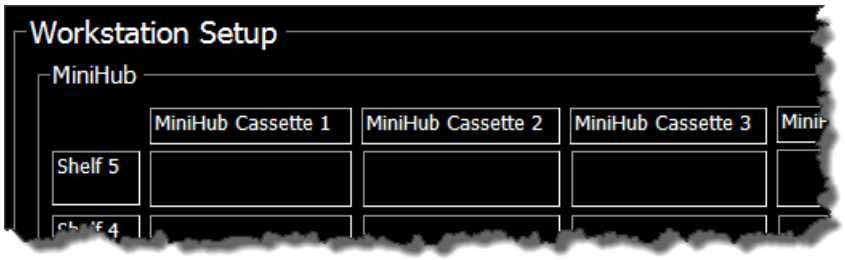
31 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

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

33 Click **Display Initial Workstation Setup**.



34 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

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

NOTE

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

Proceed directly to **"Step 3. Prepare adaptor-ligated libraries"** on page 47.

Step 2, Option 2. Prepare fragmented DNA by mechanical shearing

The SureSelect^{XT} Low Input target enrichment workflow supports two different options for preparing fragmented DNA.

- Option 1 uses enzymatic fragmentation, with liquid handling steps executed by automation protocols, and is described in **"Step 2, Option 1. Prepare fragmented DNA by enzymatic fragmentation"** on page 38.
- Option 2, which is described in this section, uses mechanical shearing.

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

In the option described in this section (option 2), the 50-µL gDNA samples are mechanically sheared using conditions optimized for either high-quality or FFPE DNA. The target DNA fragment size is 150 to 200 bp.

NOTE

This protocol has been optimized using a Covaris model E220 instrument and the Covaris 96 microTUBE plate for a target DNA fragment size of 150 to 200 bp. If you wish to use a different Covaris instrument model/sample holder or if your NGS workflow requires a different DNA fragment size (e.g., for translocation detection with the SureSelect Cancer All-In-One assay), consult the manufacturer's recommendations for shearing conditions for the recommended DNA fragment size.

1 Set up the Covaris E220 instrument. Refer to the Covaris instrument user guide for details.
   - **a** Check that the water in the Covaris tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate in use.
   - **b** Check that the water covers the visible glass part of the tube.
   - **c** On the instrument control panel, push the Degas button. Degas the instrument according to the manufacturer's recommendations, typically 30–60 minutes.
   - **d** Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C. Consult the manufacturer's recommendations for addition of coolant fluids to prevent freezing.
2 Prepare the DNA samples for the run by diluting 10–200 ng of each gDNA sample with 1X Low TE Buffer (10 mM Tris- HCl, pH 7.5–8.0, 0.1 mM EDTA) to a final volume of 50 µL. Vortex well to mix, then spin briefly to collect the liquid. Keep the samples on ice.

   See **Table 12** or **Table 13** for FFPE DNA input guidelines based on the measured DNA quality in each sample.

NOTE

Do not dilute samples to be sheared using water. Shearing samples in water reduces the overall library preparation yield and complexity.

3 Vortex each sample dilution to mix, then spin briefly to collect the liquid. Keep the samples on ice.
4 Complete the DNA shearing steps below for each of the gDNA samples.

   Transfer the 50-µL DNA sample into a Covaris 96 microTUBE Plate, using a tapered pipette tip to slowly transfer the sample through the pre-split foil seal. Seal the plate with the provided foil seal.
   - **e** Spin the 96 microTUBE Plate for 30 seconds to collect the liquid and to remove any bubbles from the bottom of the tube.

f Load the 96 microTUBE Plate onto the loading tray and shear the DNA with the settings in **Table 24**.

Table 24 Shear settings for Covaris E-series instrument (SonoLab software v7 or later)

Setting	High-quality DNA	FFPE DNA
Duty Factor	10%	10%
Peak Incident Power (PIP)	175	175
Cycles per Burst	200	200
Treatment Time	2 × 120 seconds	240 seconds
Bath Temperature	2° to 8° C	2° to 8° C

Use the steps below for two-round shearing of high-quality DNA samples only:

- Shear for 120 seconds
- Spin the 96 microTUBE Plate for 10 seconds
- Shear for additional 120 seconds
- Spin the 96 microTUBE Plate for 10 seconds

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

h Transfer the sheared DNA sample (approximately 50 µL) to a Eppendorf twin.tec 96-well plate sample well. Keep the samples on ice.

i After transferring the DNA sample, spin the 96 microTUBE Plate briefly to collect any residual sample volume. Transfer any additional collected liquid to the sample well used in **step h**.

NOTE It is important to avoid loss of input DNA at this step, especially for low-abundance DNA samples. Visually inspect the microTUBE to ensure that all of the sample has been transferred. If droplets remain in the microTUBE, repeat **step i**.

Step 3. 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 or P5 indexed 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 25**. Thaw and mix each component as directed in **Table 25** 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 AMPure XP beads, which must be removed from cold storage and equilibrated to room temperature before use on **page 51**.

Table 25 Reagents thawed before use in protocol

Kit Component	Storage Location	Thawing Conditions	Mixing Method	Where Used
End Repair-A Tailing Buffer (bottle)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	**page 49**
Ligation Buffer (bottle)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice (may require >20 minutes) then keep on ice	Vortexing	**page 49**
End Repair-A Tailing Enzyme Mix (orange cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Place on ice just before use	Inversion	**page 49**
T4 DNA Ligase (blue cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Place on ice just before use	Inversion	**page 49**
Adaptor Oligo Mix (white cap) **-- needed for Single Indexing runs only** *or* P5 Indexed Adaptors (green plate) **-- needed for Dual Indexing runs only**	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C *or* SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM	Thaw on ice then keep on ice	Vortexing	**page 50**

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 20°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.

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

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

5 Prepare the appropriate volume of End Repair/dA-Tailing master mix, using volumes listed in **Table 26** 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 for 15–30 seconds to ensure homogeneity.) Spin briefly to collect the liquid and keep on ice.

Table 26 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 (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

6 Prepare the appropriate volume of Ligation master mix, using volumes listed in **Table 27** 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 for 15–30 seconds to ensure homogeneity.) Spin briefly to collect the liquid.

Table 27 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 (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 *OR* the P5 indexed adaptor plate

7 For single-indexed libraries, follow the instructions in part **a** below. For dual-indexed libraries, follow instructions in part **b**.

a **Do this step only when preparing single (P7)-indexed libraries**. Prepare the appropriate volume of Adaptor Oligo Mix dilution, according to **Table 28**. Mix well using a vortex mixer and keep on ice.

Table 28 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	266.5 µL
Adaptor Oligo Mix (white cap)	5 µL	85.0 µL	127.5 µL	170.0 µL	212.5 µL	287.0 µL	552.5 µL
Total Volume	**7.5 µL**	**127.5 µL**	**191.3 µL**	**255.0 µL**	**318.8 µL**	**430.5 µL**	**819 µL**

b **Do this step only when preparing dual-indexed libraries**. Prepare the P5 indexed adaptor source plate using a fresh Eppendorf twin.tec plate. Place 2.5 µL of nuclease free water into each sample well of the plate then add 5 µL of each P5 indexed-adaptor to be used in the run into its assigned plate well.

Prepare the master mix source plate

8 In a Nunc DeepWell plate, prepare the master mix source plate containing the mixtures prepared in **step 5** through **step 7**. Add the volumes indicated in **Table 29** of each mixture to all wells of the indicated column of the Nunc DeepWell 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 positions of the master mixes on the source plate have changed since the previous revision of the LibraryPrep_XT_LI_ILM runset (LibraryPrep_XT_LI_ILM_v.B1.1.1.rst) in that column 1 now remains empty. Make sure to refer to **Figure 5** for the correct source plate configuration.

Table 29 Preparation of the Master Mix Source Plate for LibraryPrep_XT_LI_ILM runset

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
End Repair-dA Tailing master mix	Column 2 (A2-H2)	31.0 µL	52.0 µL	73.0 µL	94.0 µL	136.0 µL	283.0 µL
Ligation master mix	Column 3 (A3-H3)	36.0 µL	62.0 µL	88.0 µL	114.0 µL	166.0 µL	348.0 µL
Adaptor Oligo Mix dilution **(Single Indexing runs only)**	Column 4 (A4-H4)*	15.0 µL	22.5 µL	30.0 µL	37.5 µL	52.5 µL	101.3 µL

* Leave Column 4 empty when preparing the source plate for a Dual Indexing run. The P5 indexed adaptors that replace the Adaptor Oligo Mix in these runs are supplied in a separate source plate.

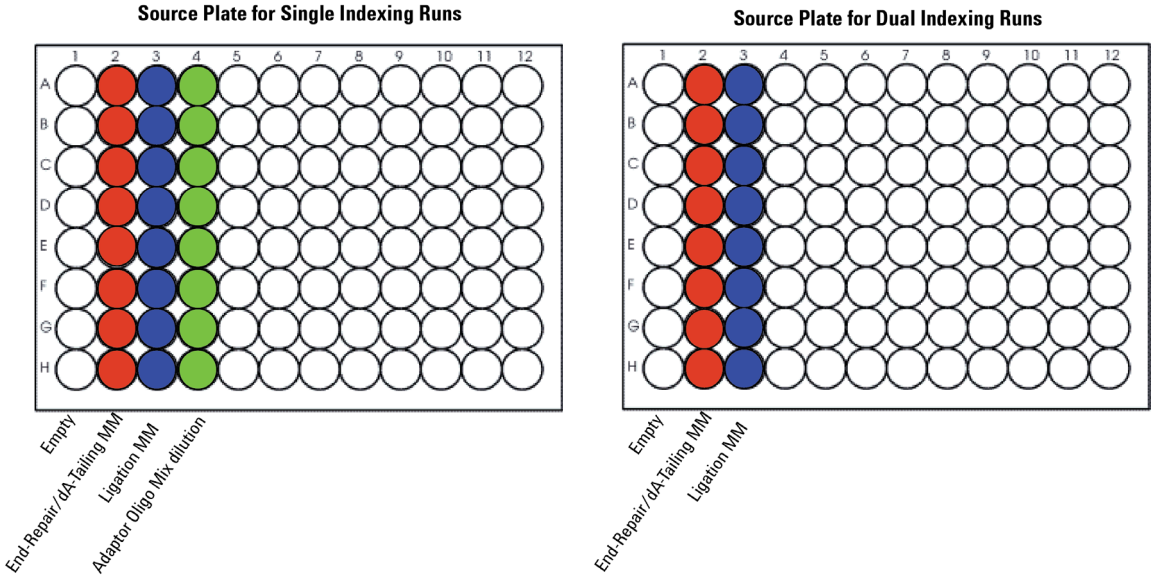


Figure 5 Configuration of the master mix source plate for the LibraryPrep_XT_LI_ILM runset in a Nunc DeepWell plate.

9 Seal the master mix 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.

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

11 Verify that the AMPure XP bead suspension is at room temperature. *Do not freeze the beads at any time.*

12 Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.

13 Prepare a separate Nunc DeepWell source plate for the beads by adding 140 µL of homogeneous AMPure XP beads per well, for each well to be processed.

NOTE The 140 µL of AMPure XP beads dispensed in this step is used in both the LibraryPrep_XT_LI_ILM runset and subsequent AMPureXP_XT_LI_ILM purification protocol. If the library preparation runset and purification protocol are not run on the same day, be sure to seal the AMPure XP bead source plate and store at 4°C.

14 Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water.

15 Prepare a separate Thermo Scientific reservoir containing 45 mL of freshly-prepared 70% ethanol.

Load the Agilent NGS Workstation

16 Load the Labware MiniHub according to **Table 30**, using the plate orientations shown in **Figure 4** on page 41.

Table 30 Initial MiniHub configuration for LibraryPrep_XT_LI_ILM runset

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	—	—	—
Shelf 4	Empty Eppendorf twin.tec plate (Single indexing) **OR** Diluted P5 indexed adaptors in Eppendorf twin.tec plate (Dual indexing)	—	—	—
Shelf 3	Empty Eppendorf twin.tec plate	Empty Eppendorf twin.tec plate	—	—
Shelf 2	New tip box	Nuclease-free water reservoir from **step 14**	AMPure XP beads in Nunc DeepWell plate from **step 13**	—
Shelf 1 (Bottom)	Empty tip box	70% ethanol reservoir from **step 15**	—	Empty tip box

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

Table 31 Initial Bravo deck configuration for LibraryPrep_XT_LI_ILM runset

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
4	Empty red insert
6	Empty Eppendorf twin.tec plate
7	Eppendorf plate containing sheared gDNA samples
9	Library Prep Master Mix Source Plate, unsealed

18 Load the BenchCel Microplate Handling Workstation according to **Table 32**.

Table 32 Initial BenchCel configuration for LibraryPrep_XT_LI_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	8 Tip boxes	—	—	—

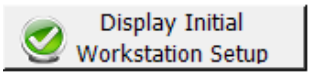
Run VWorks runset LibraryPrep_XT_LI_ILM

19 On the SureSelect setup form, under **Select Protocol to Run,** select the **LibraryPrep_XT_LI_ILM** runset.

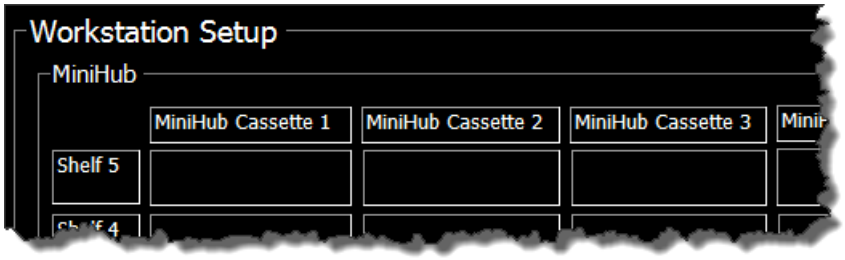
20 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

21 Select the indexing type (Single or Dual) according to the configuration chosen in **step 7**.

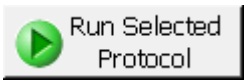
22 Click **Display Initial Workstation Setup**.



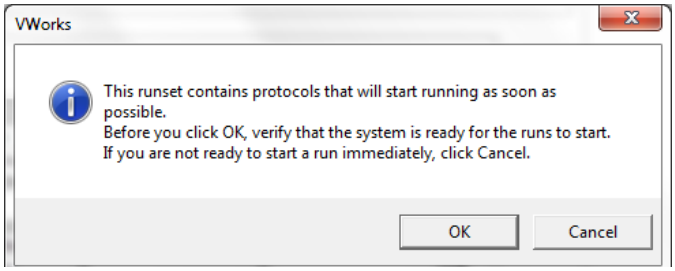
23 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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



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



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

Stopping Point If you do not continue to the next step, seal the DNA sample plate and store at 4°C overnight or at –20°C for prolonged storage. Remove the Nunc DeepWell source plate containing AMPure XP beads from the Cassette 3, Shelf 2 of the Labware MiniHub; seal the plate and store at 4°C.

Step 4. Amplify adaptor-ligated libraries

In this step, the Agilent NGS Workstation completes the liquid handling steps for amplification and P7-indexing of the adaptor-ligated DNA 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 33**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 33 Reagents for pre-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Pipette up and down 15–20 times	**page 56**
5× Herculase II Reaction Buffer (clear cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	**page 56**
100 mM dNTP Mix (green cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	**page 56**
Forward Primer (brown cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	**page 56**
SureSelect XT Low Input Index Primers	SureSelect XT Low Input Index Primers for ILM (Pre PCR),* –20°C	Vortexing	**page 56**

* Indexing primers are provided in 96-well plates containing either indexes 1–96 (yellow *Index Plate 1*) or indexes 97–192 (red *Index Plate 2*). If dual-indexed primers are used, ensure that they are paired with the correct P7 primer from Index Plate 1.

NOTE Take care to avoid combining libraries with the same index sequence when multiplexing libraries prepared using different SureSelect kit formats. For example, indexes 1-32 in SureSelect XT Low Input Reagent Kits (provided in yellow plate) are equivalent to indexes A01-H04 in SureSelect XT HS Reagent Kits (provided in black capped-tubes) and to indexes A01-H04 in Magnis SureSelect XT HS automation kits (provided in black index strips).

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. Retain the Nunc DeepWell source plate containing AMPure XP beads in the Labware MiniHub Cassette 3, Shelf 2 for use in a later purification 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. 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

5. Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 34**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the automation run.

Table 34 Pre-Capture PCR Thermal Cycler Program*

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8 to 14, based on input DNA quality and quantity (see **Table 35**)	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

* When setting up the thermal cycling program, use a reaction volume setting of 50 µl.

Table 35 Pre-capture PCR cycle number recommendations

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

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

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

6 Prepare the appropriate volume of pre-capture PCR Master Mix, according to **Table 36**. Mix well using a vortex mixer and keep on ice.

Table 36 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
Nuclease-free water	9.5 µL	121.1 µL	201.9 µL	282.6 µL	363.4 µL	524.9 µL	1051.7 µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1107 µL
100 mM dNTP Mix (green cap)	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	27.6 µL	55.4 µL
Forward Primer (brown cap)	2 µL	25.5 µL	42.5 µL	59.5 µL	76.5 µL	110.5 µL	221.0 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.7 µL
Total Volume	**23.0 µL**	**293.3 µL**	**488.8 µL**	**684.3 µL**	**879.8 µL**	**1270.8 µL**	**2545.8 µL**

7 Using the same Nunc DeepWell master mix source plate that was used for the LibraryPrep_XT_LI_ILM run, add the volume of PCR Master Mix indicated in **Table 37** to all wells of column 5 of the master mix source plate. The final configuration of the master mix source plate is shown in **Figure 6**.

Table 37 Preparation of the Master Mix Source Plate for Pre-CapPCR_XT_LI_ILM protocol

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Pre-Capture PCR Master Mix	Column 5 (A5-H5)	34.5 µL	57.5 µL	80.5 µL	103.5 µL	149.5 µL	310.5 µL

CAUTION

Make sure to add the Pre-Capture PCR Master Mix to column 5 of the source plate. Column 4 remains empty for dual indexing runs (see Figure 5).

If you are using a new DeepWell plate for the pre-capture PCR source plate, leave columns 1 to 4 empty and add the PCR Master Mix to column 5 of the new plate.

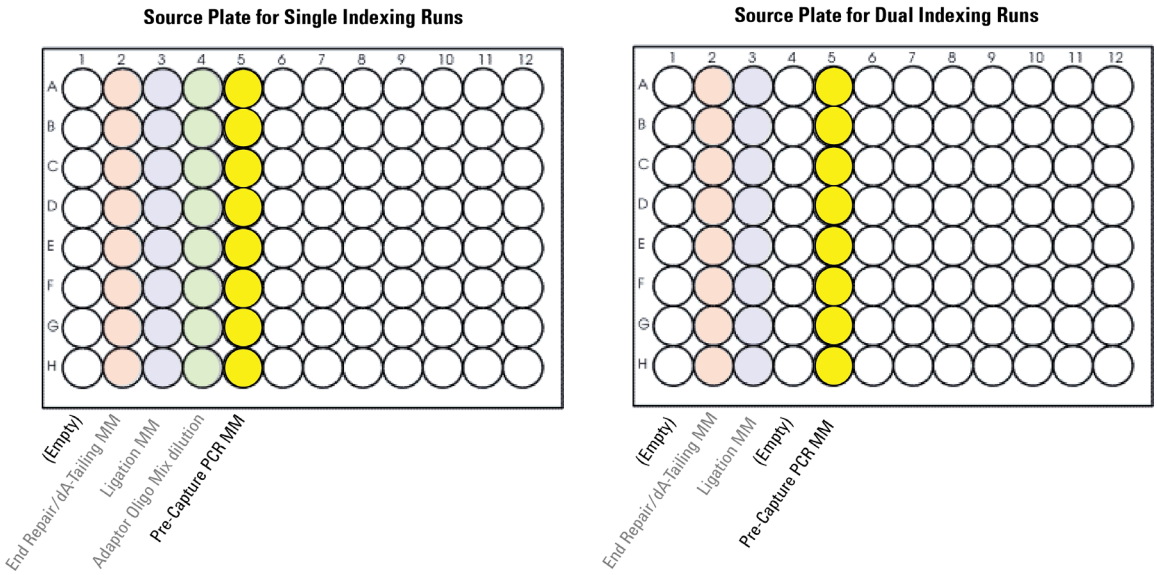


Figure 6 Configuration of the master mix source plate for the Pre-CapPCR_XT_LI_ILM protocol. Master mixes dispensed during previous protocols are shown in light shading.

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

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

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 pre-capture PCR indexing primer plate

10 Prepare the SureSelect XT Low Input Index Primers in the PCR plate to be used for the amplification automation protocol. In each well of the PCR plate, place 2 µL of the specific indexing primer assigned to the sample well. Keep the plate on ice.

Load the Agilent NGS Workstation

11 Load the Labware MiniHub according to **Table 38**, using the plate orientations shown in **Figure 4** on page 41.

Table 38 Initial MiniHub configuration for Pre-CapPCR_XT_LI_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	—	AMPure XP beads in Nunc DeepWell plate (retained from previous runset-not used in this protocol)	—
Shelf 1 (Bottom)	Empty tip box	—	—	Empty tip box

12 Load the Bravo deck according to **Table 39**.

Table 39 Initial Bravo deck configuration for Pre-CapPCR_XT_LI_ILM protocol

Location	Content
6	SureSelect XT Low Input Index Primers in PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
7	Adaptor-ligated DNA samples in Eppendorf twin.tec plate
9	Master mix plate containing PCR Master Mix in Column 5 (unsealed)

13 Load the BenchCel Microplate Handling Workstation according to **Table 40**.

Table 40 Initial BenchCel configuration for Pre-CapPCR_XT_LI_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_LI_ILM

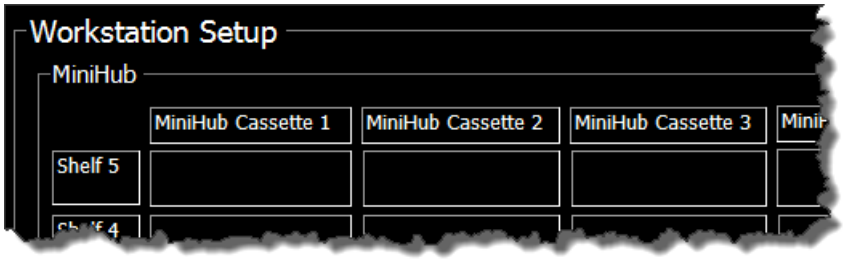
14 On the SureSelect setup form, under **Select Protocol to Run**, select the **Pre-CapPCR_XT_LI_ILM** protocol.

15 Under **Select PCR plate 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**.

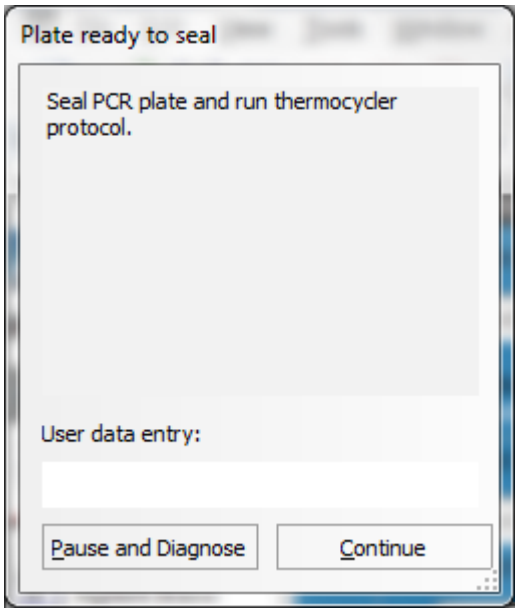
18 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

Running the Pre-CapPCR_XT_LI_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.

20 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 3.0 seconds.



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

22 Before adding the samples to the preprogrammed thermal cycler, bring the temperature of the thermal block to 98°C by resuming the thermal cycling program in **Table 34**. 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 5. Purify amplified DNA using AMPure XP beads

In this step, the Agilent NGS Workstation transfers AMPure XP beads and amplified DNA to a Nunc DeepWell plate and then collects and washes the bead-bound DNA.

Prepare the workstation and reagents

1 Retain the Nunc DeepWell source plate containing AMPure XP beads in the Labware MiniHub Cassette 3, Shelf 2 for use in this protocol. Otherwise, clear the Labware MiniHub and BenchCel of all plates and tip boxes.

CAUTION To prevent damage to the Agilent NGS Workstation, ensure that the Nunc DeepWell plate is unsealed before you proceed.

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 a Thermo Scientific reservoir containing 15 mL of nuclease-free water.

6 Prepare a separate Thermo Scientific reservoir containing 45 mL of freshly-prepared 70% ethanol.

7 Load the Labware MiniHub according to **Table 41**, using the plate orientations shown in **Figure 4** on page 41.

Table 41 Initial MiniHub configuration for AMPureXP_XT_LI_ILM (Pre-Capture PCR) protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	Empty Eppendorf twin.tec Plate	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 3**	AMPure XP beads in Nunc DeepWell plate (unsealed; retained from earlier runset)	—
Shelf 1 (Bottom)	—	70% ethanol reservoir from **step 6**	—	Empty tip box

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

Table 42 Initial Bravo deck configuration for AMPureXP_XT_LI_ILM (Pre-Capture PCR) protocol

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
9	Amplified DNA libraries in unsealed PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)

9 Load the BenchCel Microplate Handling Workstation according to **Table 43**.

Table 43 Initial BenchCel configuration for AMPureXP_XT_LI_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	6 Tip boxes	—	—	—

Run VWorks protocol *AMPureXP_XT_LI_ILM* (Pre-Capture PCR)

10 On the SureSelect setup form, under **Select Protocol to Run**, select the **AMPureXP_XT_LI_ILM (Pre-Capture PCR)** protocol.

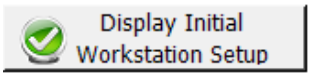
NOTE

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.

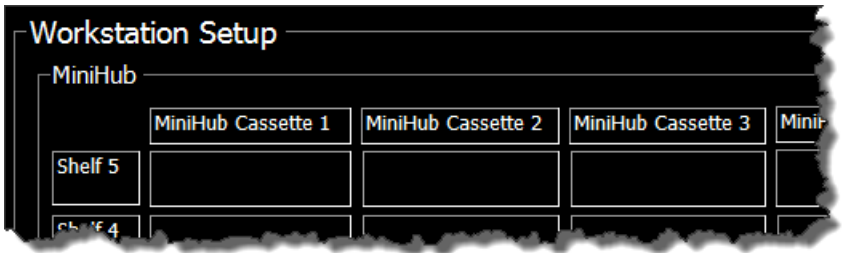
11 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate containing the amplified libraries at position 9.

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

13 Click **Display Initial Workstation Setup**.



14 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

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

Step 6. Assess Library DNA quantity and quality

Sample analysis can be completed using one of two options.

- Option 1: Prepare the analytical assay plate using automation (protocol TS_D1000) and perform analysis on Agilent 4200 TapeStation. See **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape"** on page 63.
- 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 67.

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 a fresh Eppendorf twin.tec plate, prepare the Sample Buffer source plate at room temperature. Add the volume of D1000 Sample Buffer indicated in **Table 44** to each well of column 1 of the plate.

Table 44 Preparation of the Sample Buffer Source Plate for TS_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Eppendorf twin.tec Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
D1000 Sample Buffer	Column 1 (A1-H1)	11.0 µL	17.0 µL	23.0 µL	29.0 µL	41.0 µL	77.0 µL

Load the Agilent NGS Workstation

6 Load the Labware MiniHub according to **Table 45**, using the plate orientations shown in **Figure 4** on page 41.

Table 45 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 46**.

Table 46 Initial Bravo deck configuration for TS_D1000 protocol

Location	Content
4	Amplified pre-capture libraries in Eppendorf twin.tec plate (unsealed)
6	Empty Eppendorf twin.tec plate
9	Eppendorf twin.tec source plate containing D1000 Sample Buffer in Column 1

CAUTION

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

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

8 Load the BenchCel Microplate Handling Workstation according to **Table 47**.

Table 47 Initial BenchCel configuration for TS_D1000 protocol

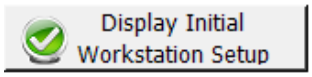
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Run VWorks protocol TS_D1000

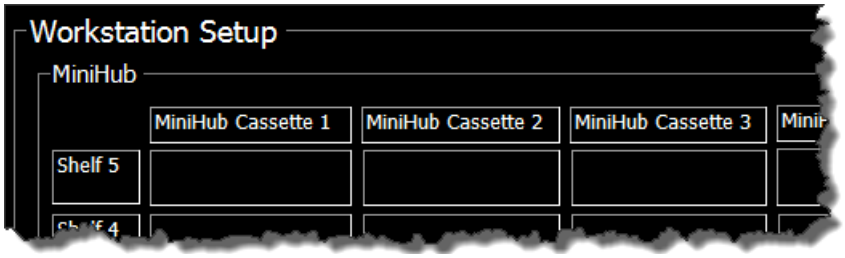
9 On the SureSelect setup form, under **Select Protocol to Run**, select **TS_D1000**.

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**.



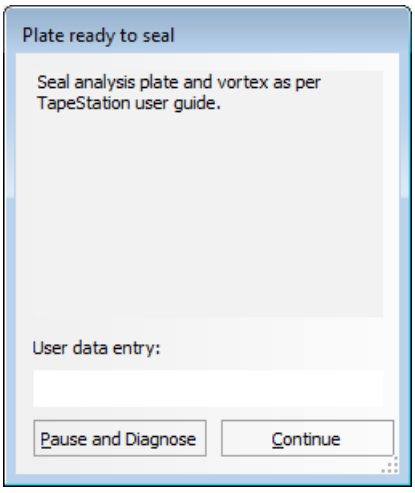
12 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

Running the TS_D1000 protocol takes approximately 15 minutes. Once removal of analytical samples is complete, remove the primary DNA library sample plate from position 4, seal the plate, and keep on ice until the samples are used for hybridization set up on **page 71**.

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



CAUTION

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

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

Run the D1000 Assay and analyze the data

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

16 Verify that the electropherogram shows the peak of DNA fragment size positioned between 300 to 400 bp for high-quality DNA and approximately 200 to 400 bp for FFPE DNA. Sample electropherograms are shown in **Figure 7** (library prepared from high-quality DNA), **Figure 8** (library prepared from medium-quality FFPE DNA), and **Figure 9** (library prepared from low-quality FFPE DNA).

The appearance of an additional low molecular weight peak indicates the presence of adaptor-dimers in the library. It is acceptable to proceed to target enrichment with library samples for which adaptor-dimers are observed in the electropherogram at low abundance, similar to that seen in sample electropherograms below. See Troubleshooting information on **page 138** for additional considerations.

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

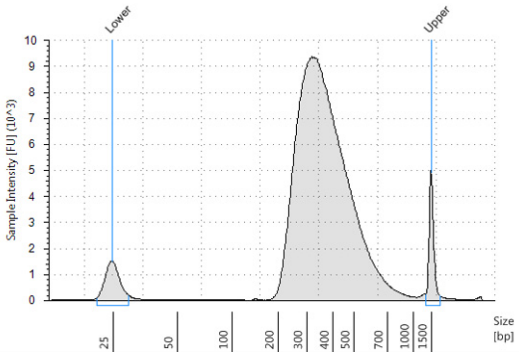


Figure 7 Pre-capture library prepared from a high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

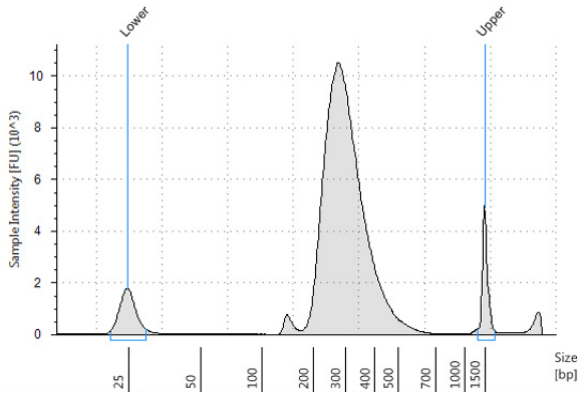


Figure 8 Pre-capture library prepared from a typical FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

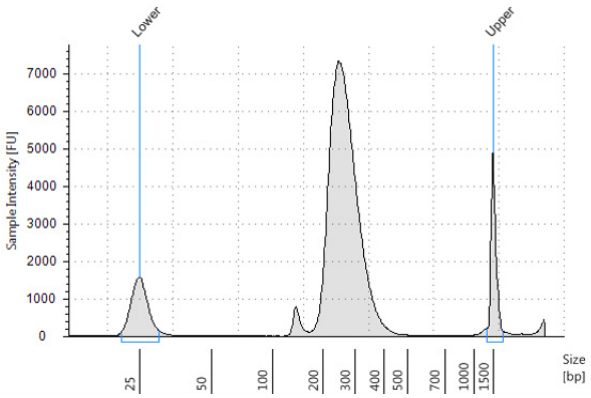


Figure 9 Pre-capture library prepared from a low-quality FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

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

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

Using manual preparation of the analytical sample plate, you can analyze the DNA samples on Agilent 2100 BioAnalyzer, Agilent 5200 Fragment Analyzer, or Agilent 4200/4150 TapeStation. Electropherograms obtained using one of these other analysis platforms are expected to show fragment size profiles similar to those shown for the Agilent 4200 TapeStation (see **Figure 7** through **Figure 9**). **Table 48** includes links to assay instructions.

Table 48 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.

4 Hybridization

This chapter describes the steps to complete the hybridization and capture steps using a SureSelect or ClearSeq Capture Library. Each DNA library sample must be hybridized and captured individually.

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

Step 1. Aliquot prepped DNA samples for hybridization

For each sample library prepared, do one hybridization and capture. Do not pool samples at this stage.

The hybridization reaction requires 500–1000 ng of prepared DNA in a volume of 12 µL. Use the maximum amount of prepared DNA available within this range.

Using the DNA concentration for each sample determined on **page 63** to **page 67**, calculate the volume of each sample to be used for hybridization using the formula below:

Volume (µL) = 1000 ng/concentration (ng/µL)

If the concentration of any sample is not sufficient to allow use of the recommended 1000 ng of DNA, use the full remaining volume of DNA sample (approximately 10 to 12 µL, containing at least 500 ng) for the hybridization step.

The automation protocol Aliquot_Libraries is used to prepare a new sample plate containing the appropriate volume of each DNA sample for hybridization. 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 10**. 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 70** for guidelines). For all empty wells on the plate, enter the value 0, as shown in **Figure 10**; do not delete rows for empty wells.

	A	B	C	D
1	**SourceBC**	**SourceWell**	**DestinationWell**	**Volume**
2	SamplePlateXYZ	A1	A1	5.35
3	SamplePlateXYZ	B1	B1	4.28
4	SamplePlateXYZ	C1	C1	4.76
5	SamplePlateXYZ	D1	D1	5.19
6	SamplePlateXYZ	E1	E1	5.49
7	SamplePlateXYZ	F1	F1	4.86
8	SamplePlateXYZ	G1	G1	5.05
9	SamplePlateXYZ	H1	H1	4.37
10	SamplePlateXYZ	A2	A2	0
11	SamplePlateXYZ	B2	B2	0
12	SamplePlateXYZ	C2	C2	0
13	SamplePlateXYZ	D2	D2	0

Figure 10 Sample spreadsheet for 1-column run.

NOTE

You can find a sample spreadsheet in the directory **C: > VWorks Workspace > NGS Option B > XT_LI_ILM_v.Bx.x.x> Aliquot Library Input Files > Aliquot_Libraries_template.csv** (where x.x.x is the version number of the XT_LI_ILM form).

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 retain rows for all 96 wells, and populate the Volume column with 0 for unused wells.

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

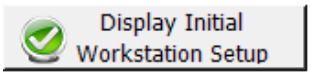
5 Load the Bravo deck according to **Table 49**.

Table 49 Initial Bravo deck configuration for Aliquot_Libraries protocol

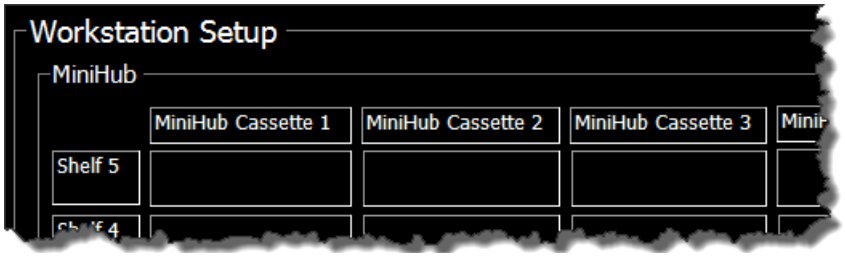
Location	Content
5	Empty Eppendorf twin.tec plate
6	Empty tip box
8	New tip box
9	Prepped library DNA in Eppendorf twin.tec plate

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

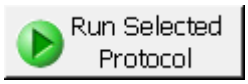
7 Click **Display Initial Workstation Setup**.



8 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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



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

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 and use a vacuum concentrator to dry the samples at ≤ 45°C.

12 Reconstitute each dried sample with 12 µL of nuclease-free water. Pipette up and down along the sides of each well for optimal recovery.

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

14 Vortex the plate for 30 seconds to ensure complete reconstitution, then centrifuge the plate for 1 minute to drive the well contents off the walls and plate seal.

Step 2. Hybridize the gDNA library and probe

In this step, automation protocol Hyb_XT_LI_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 50**. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 50 Reagents for Hybridization

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect XT HS and XT Low Input Blocker Mix (blue cap)	SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	**page 74**
SureSelect RNase Block (purple cap)	SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw on ice	**page 75**
SureSelect Fast Hybridization Buffer (bottle)	SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR), –20°C	Thaw and keep at Room Temperature	**page 74**
Probe Capture Library	–80°C	Thaw on ice	**page 75**

Program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 51**. 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 51 Pre-programmed thermal cycler program for Hybridization*

Segment Number	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 cycling 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 82**.

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

Prepare the workstation

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

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

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

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

6 Place an empty tip box on shelf 2 of cassette 4 of the workstation MiniHub.

7 Load tip boxes for the run in the BenchCel Microplate Handling Workstation according to Table 52.

Table 52 Initial BenchCel configuration for Hyb_XT_LI_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	3 Tip boxes	—	—	—
6	3 Tip boxes	—	—	—
12	4 Tip boxes	—	—	—

Prepare the Block master mix

8 Prepare the appropriate volume of Block master mix, on ice, as indicated in Table 53.

Table 53 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 HS and XT Low Input Blocker Mix (blue cap)	5.0 µL	63.8 µL	106.3 µL	148.8 µL	191.3 µL	276.3 µL	552.5 µL
Total Volume	**7.5 µL**	**95.7 µL**	**159.4 µL**	**223.2 µL**	**286.9 µL**	**414.4 µL**	**828.8 µL**

Prepare the Hybridization Buffer master mix

9 Prepare the appropriate volume of Hybridization Buffer master mix, at room temperature, as indicated in Table 54.

Table 54 Preparation of Hybridization Buffer 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	53.1 µL	74.4 µL	95.6 µL	116.9 µL	159.4 µL	297.5 µL
SureSelect Fast Hybridization Buffer (bottle)	6.0 µL	127.5 µL	178.5 µL	229.5 µL	280.5 µL	382.5 µL	714.0 µL
Total Volume	**8.5 µL**	**180.6 µL**	**252.9 µL**	**325.1 µL**	**397.4 µL**	**541.9 µL**	**1011.5 µL**

Prepare one or more Capture Library Master Mixes

10 Prepare the appropriate volume of Capture Library Master Mix for each of the Probes that will be used for hybridization as indicated in **Table 55** to **Table 58**. Mix thoroughly by vortexing at high speed then spin down briefly. Keep the Capture Library Master Mix(es) on ice.

NOTE

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

For runs that use a single Probe for all rows of the plate, prepare the master mix as described in Step a (**Table 55** or **Table 56**) on **page 75**.

For runs that use different Probes for individual rows, prepare each master mix as described in Step b (**Table 57** or **Table 58**) on **page 76**.

a For runs that use a single Probe for all rows, prepare a Master Mix as described in **Table 55** or **Table 56**, according to the probe design size.

Table 55 Preparation of Capture Library 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	4.5 µL	76.5 µL	114.8 µL	153.0 µL	191.3 µL	306.0 µL	592.9 µL
RNase Block (purple cap)	0.5 µL	8.5 µL	12.8 µL	17.0 µL	21.3 µL	34.0 µL	65.9 µL
Probe Capture Library	2.0 µL	34.0 µL	51.0 µL	68.0 µL	85.0 µL	136.0 µL	263.5 µL
Total Volume	**7.0 µL**	**119.0 µL**	**178.6 µL**	**238.0 µL**	**297.6 µL**	**476.0 µL**	**922.3 µL**

Table 56 Preparation of Capture Library 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	1.5 µL	25.5 µL	38.3 µL	51.0 µL	63.8 µL	102.0 µL	197.6 µL
RNase Block (purple cap)	0.5 µL	8.5 µL	12.8 µL	17.0 µL	21.3 µL	34.0 µL	65.9 µL
Probe Capture Library	5.0 µL	85.0 µL	127.5 µL	170.0 µL	212.5 µL	340.0 µL	658.8 µL
Total Volume	**7.0 µL**	**119.0 µL**	**178.6 µL**	**238.0 µL**	**297.6 µL**	**476.0 µL**	**922.3 µL**

b For runs that use different Probes in individual rows, prepare a Master Mix for each Probe as listed in **Table 57** or **Table 58**, according to the probe design size. The volumes listed in **Table 57** and **Table 58** 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 57 Preparation of Capture Library 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	4.5 µL	9.0 µL	13.8 µL	18.6 µL	23.3 µL	37.7 µL	73.5 µL
RNase Block (purple cap)	0.5 µL	1.0 µL	1.5 µL	2.1 µL	2.6 µL	4.2 µL	8.2 µL
Probe Capture Library	2.0 µL	4.0 µL	6.1 µL	8.3 µL	10.4 µL	16.8 µL	32.7 µL
Total Volume	**7.0 µL**	**14.0 µL**	**21.4 µL**	**29.0 µL**	**36.3 µL**	**58.7 µL**	**114.4 µL**

Table 58 Preparation of Capture Library 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	1.5 µL	3.0 µL	4.6 µL	6.2 µL	7.8 µL	12.6 µL	24.5 µL
RNase Block (purple cap)	0.5 µL	1.0 µL	1.5 µL	2.1 µL	2.6 µL	4.2 µL	8.2 µL
Probe Capture Library	5.0 µL	10.0 µL	15.3 µL	20.6 µL	25.9 µL	41.9 µL	81.7 µL
Total Volume	**7.0 µL**	**14.0 µL**	**21.4 µL**	**28.9 µL**	**36.3 µL**	**58.7 µL**	**114.4 µL**

Prepare the master mix source plate

11 Using an Eppendorf twin.tec plate, prepare the hybridization master mix source plate at room temperature, containing the master mixes prepared in **step 8** to **step 10**. Add the volumes indicated in **Table 59** of each master mix to each well of the indicated column of the plate. When using multiple Probes in a run, add the Capture Library master mix for each probe to the appropriate row(s) of the Eppendorf twin.tec plate. The final configuration of the master mix source plate is shown in **Figure 11**.

Table 59 Preparation of the Master Mix Source Plate for Hyb_XT_LI_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**
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
Capture Library master mix	Column 2 (A2-H2)	14.0 µL	21.4 µL	28.9 µL	36.3 µL	58.6 µL	114.4 µL
Hybridization Buffer master mix	Column 3 (A3-H3)	19.9 µL	29.0 µL	38.0 µL	47.0 µL	65.1 µL	123.8 µL

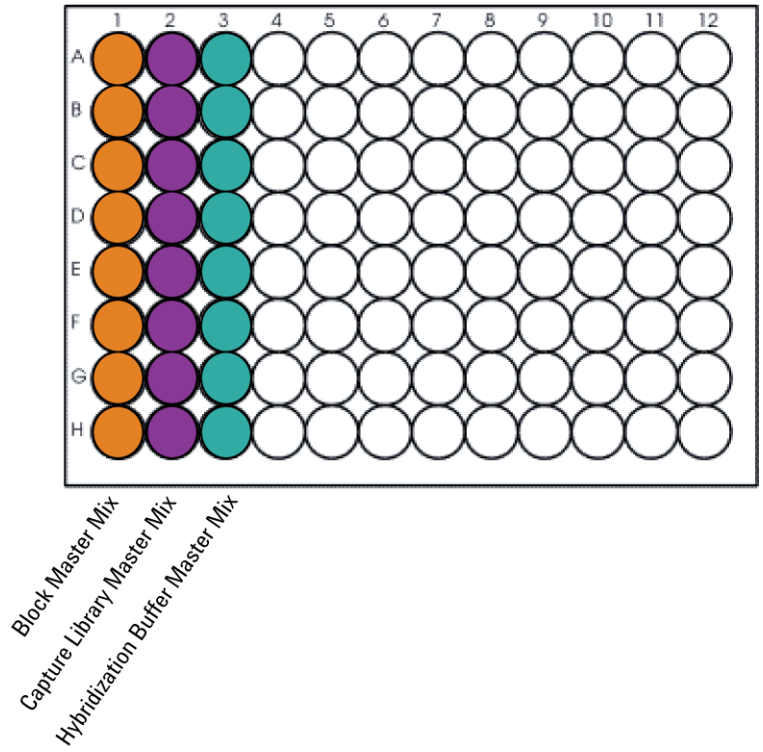


Figure 11 Configuration of the master mix source plate for Hyb_XT_LI_ILM protocol in an Eppendorf twin.tec plate. Column 2 can contain different Capture Library master mixes in each row.

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

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

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

Table 60 Initial Bravo deck configuration for Hyb_XT_LI_ILM protocol

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

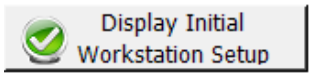
Run VWorks protocol Hyb_XT_LI_ILM

15 On the SureSelect setup form, under **Select Protocol to Run**, select the **Hyb_XT_LI_ILM** protocol.

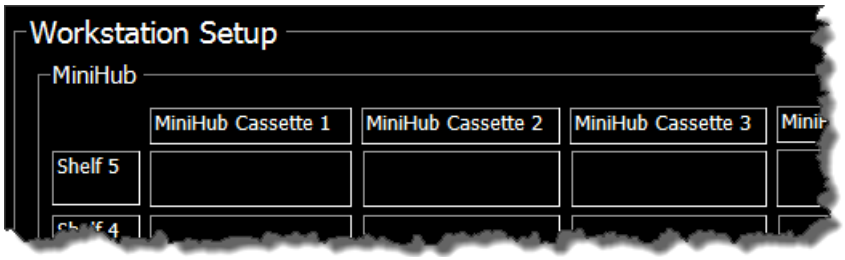
16 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 4 of the Bravo deck.

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

18 Click **Display Initial Workstation Setup**.



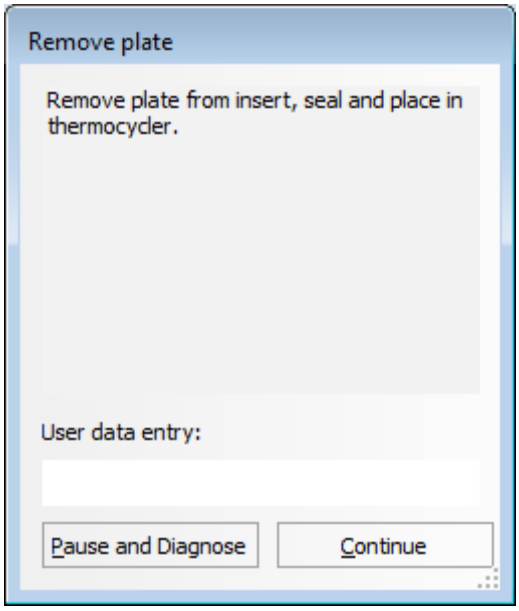
19 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

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

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



22 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec.

23 Transfer the sealed plate to a thermal cycler and initiate the preprogrammed thermal cycling program described in **Table 51** on page 73. The denaturation and blocking segments of the preprogrammed thermal cycler program are shown in **Figure 12** below for reference.

Segment Number	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

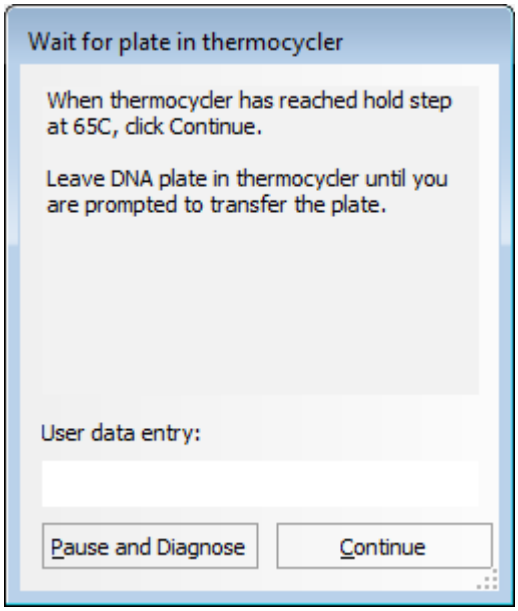
Figure 12 Preprogrammed thermal cycler segments used for sample denaturation and blocking prior to hybridization.

While the sample plate incubates on the thermal cycler, the Agilent NGS Workstation combines aliquots of the Capture Library master mix and Hybridization Buffer master mix.

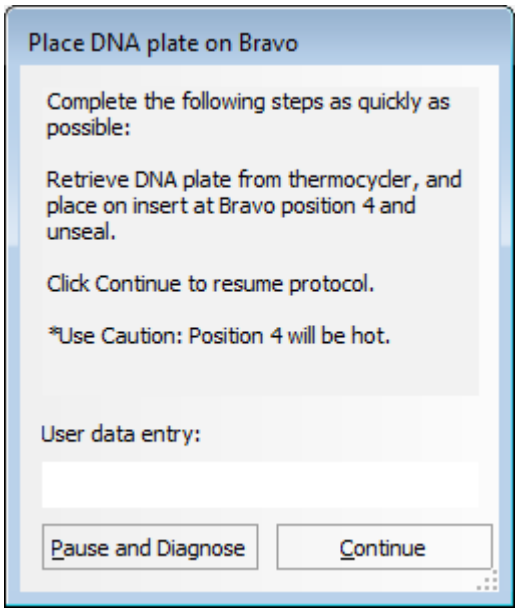
CAUTION

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

24 When the workstation has finished aliquoting the Capture Library and Hybridization Buffer master mixes, 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.



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

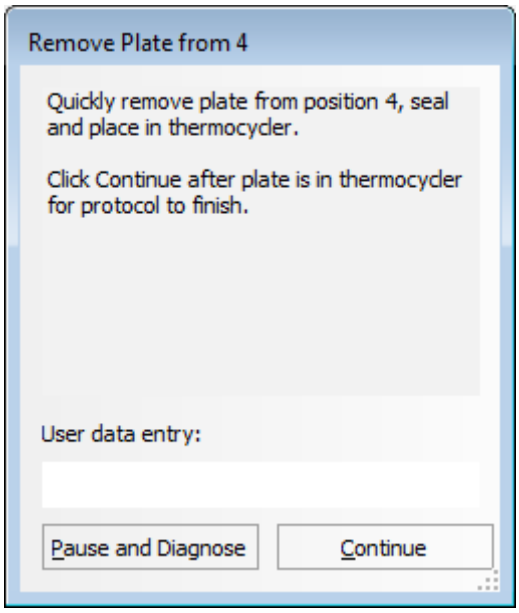


WARNING **Bravo deck position 4 will be hot.**

Use caution when handling components that contact heated deck positions.

The Agilent NGS Workstation transfers the Capture Library-hybridization buffer mixture to the wells of the PCR plate that contain the mixture of prepped gDNA samples and blocking agents.

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



27 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec.

28 Quickly transfer the plate back to the thermal cycler, held at 65°C. On the thermal cycler, initiate the hybridization segment of the pre-programmed thermal cycling program (segment 4 from **Table 51** on page 73). During this step, the prepared DNA samples are hybridized to the Probe.

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

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

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

Step 3. Capture the hybridized DNA

This step uses runset SSELCapture&Wash_XT_LI_ILM to automate capture of the gDNA-probe hybrids using streptavidin-coated magnetic beads. Setup tasks for the Capture & Wash protocol (**step 1**, below, through **step 16** on **page 84**) should be completed during the thermal cycler incubation for hybridization (approximately 1.5-hour duration) started on **page 81**.

The Capture & Wash runset uses the components listed in **Table 61**.

Table 61 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 83**
SureSelect Wash Buffer 1	SureSelect Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), RT	**page 83**
SureSelect Wash Buffer 2	SureSelect Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), RT	**page 83**
Dynabeads MyOne Streptavidin T1	Follow storage recommendations provided by supplier (see **Table 1** on page 11)	**page 83**

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. Pre-set the temperature of Bravo deck position 4 to 66°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. Place a red PCR plate insert at Bravo deck position 4.
5. Place the silver Nunc DeepWell plate insert on position 6 of the Bravo deck. This insert is required to facilitate heat transfer to DeepWell 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 Dynabeads streptavidin beads

6. Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. The beads settle during storage.
7. Wash the magnetic beads.
   a. In a conical vial, combine the components listed in **Table 62**. The volumes below include the required overage.

Table 62 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
Dynabeads MyOne Streptavidin T1 bead suspension	50 µL	425 µL	825 µL	1225 µL	1.65 mL	2.5 mL	5.0 mL
SureSelect Binding Buffer	0.2 mL	1.7 mL	3.3 mL	4.9 mL	6.6 mL	10 mL	20 mL
Total Volume	**0.25 mL**	**2.125 mL**	**4.125 mL**	**6.125 mL**	**8.25 mL**	**12.5 mL**	**25 mL**

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

c Put the vial into a magnetic separator device.

d Remove and discard the supernatant.

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

8 Resuspend the beads in SureSelect Binding buffer, according to **Table 63** below.

Table 63 Preparation of magnetic beads for SSELCapture&Wash_XT_LI_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

9 Prepare a Nunc DeepWell source plate for the washed streptavidin bead suspension. For each well to be processed, add 200 µL of the homogeneous bead suspension to the Nunc DeepWell plate.

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

Prepare capture and wash solution source plates

11 Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water.

12 Prepare an Eppendorf twin.tec source plate labeled *Wash #1*. For each well to be processed, add 160 µL of SureSelect Wash Buffer 1.

13 Prepare a Nunc DeepWell source plate labeled *Wash #2*. For each well to be processed, add 1150 µL of SureSelect Wash Buffer 2.

Load the Agilent NGS Workstation

14 Load the Labware MiniHub according to **Table 64**, using the plate orientations shown in **Figure 4** on page 41.

Table 64 Initial MiniHub configuration for SSELCapture&Wash_XT_LI_ILM runset

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

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

Table 65 Initial Bravo deck configuration for SSELCapture&Wash_XT_LI_ILM runset

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
4	Empty red insert
5	Dynabeads streptavidin bead DeepWell source plate
6	*Wash #2* DeepWell source plate seated on silver Nunc DeepWell insert

16 Load the BenchCel Microplate Handling Workstation according to **Table 66**.

Table 66 Initial BenchCel configuration for SSELCapture&Wash_XT_LI_ILM runset

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

Run VWorks runset SSELCapture&Wash_XT_LI_ILM

Start the SSELCapture&Wash_XT_LI_ILM runset upon completion of the hybridization incubation. The hybridization incubation is complete when the thermal cycler program reaches the 65°C Hold step in Segment 5 (see **Table 51** on page 73).

The total duration of the SSELCapture&Wash_XT_LI_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 67

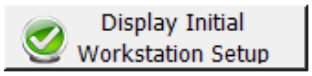
Operator action	Approximate time after run start
Transfer hybridization reactions from thermal cycler to NGS workstation	<5 minutes
Remove PCR plate from red aluminum insert	5–10 minutes

17 On the SureSelect setup form, under **Select Protocol to Run**, select the **SSELCapture&Wash_XT_LI_ILM** runset.

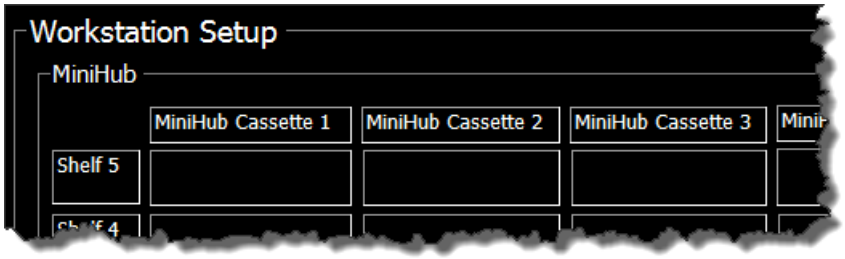
18 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used for hybridization. This plate will be transferred from the thermal cycler to Bravo deck position 4 when prompted by VWorks.

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

20 Click **Display Initial Workstation Setup**.

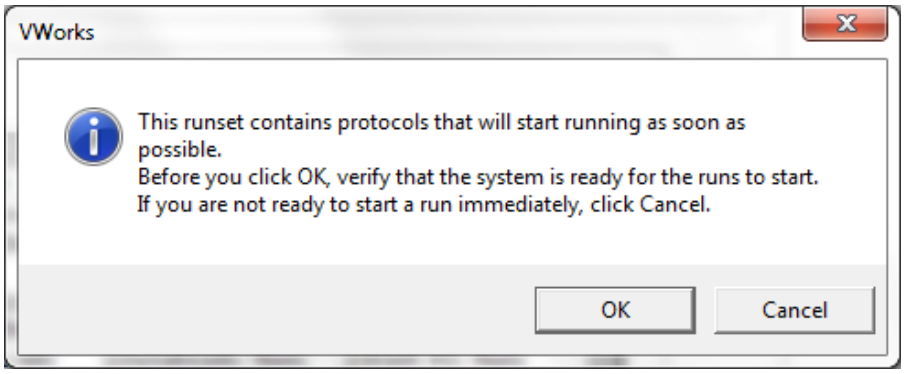


21 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



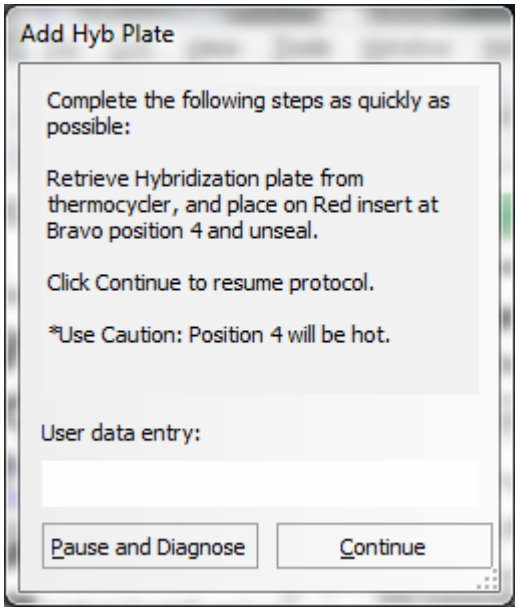
22 When verification is complete, click **Run Selected Protocol**.

23 When ready to begin the run, click **OK** in the following window. If the temperature of Bravo deck position 4 was not pre-set to 66°C, the runset will pause while position 4 reaches temperature.



CAUTION It is important to complete **step 24** quickly and carefully. Transfer the sample plate to the Bravo platform quickly to retain the 65°C sample temperature. Unseal the plate without tilting or jerking the plate to avoid sample splashing. Make sure that the Agilent NGS Workstation is completely prepared, with deck platforms at temperature and all components in place, before you transfer the sample plate to the Bravo deck.

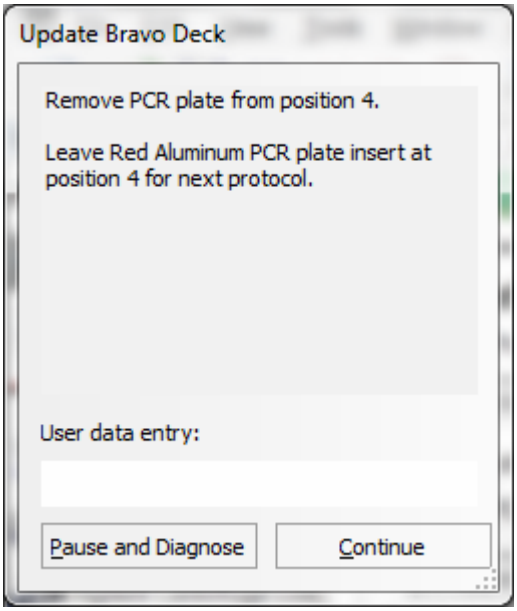
24 When prompted by VWorks as shown below, quickly remove the PCR plate, containing the hybridization reactions held at 65°C, from the thermal cycler. Unseal the plate carefully to avoid splashing, and quickly transfer the plate to position 4 of the Bravo deck, seated in the red insert. Click **Continue**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



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

Use caution when handling components that contact heated deck positions.

25 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_LI_ILM runset takes approximately 1.5 hours. Once the runset is complete, the captured, bead-bound DNA samples are located in the Eppendorf plate at position 9 of the Bravo deck.

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

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

5 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 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 Capture Library determines the amplification cycle number. Plan your experiments for amplification of samples prepared using probes of similar design sizes on the same plate. See **Table 70** on page 91 for cycle number recommendations.

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

Table 68 Reagents for post-capture PCR amplification

Component	Storage Location	Mixing Method	Where Used
Herculase II Fusion DNA Polymerase (red cap)	SureSelect XT HS and XT Low Input Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Pipette up and down 15–20 times	**page 91**
5× Herculase II Reaction Buffer (clear cap)	SureSelect XT HS and XT Low Input Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Vortexing	**page 91**
100 mM dNTP Mix (green cap)	SureSelect XT HS and XT Low Input Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Vortexing	**page 91**
SureSelect Post-Capture Primer Mix (clear cap)	SureSelect XT HS and XT Low Input Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR), –20°C	Vortexing	**page 91**

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. 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

5. Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 69**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

Table 69 Post-capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	9 to 14 See **Table 70** for recommendations based on probe design size	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

Table 70 Post-capture PCR cycle number recommendations

Probe Size/Description	Cycles
Probes <0.2 Mb	14 cycles
Probes 0.2–3 Mb (includes SSel XT HS and XT Low Input ClearSeq Comp Cancer)	12 cycles
Probes 3–5 Mb	10 cycles
Probes >5 Mb (includes Human All Exon Probes)	9 cycles

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

6 Prepare the appropriate volume of post-capture PCR Master Mix, according to **Table 71**. Mix well using a vortex mixer and keep on ice.

Table 71 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
Nuclease-free water	12.5 µL	159.4 µL	265.6 µL	371.9 µL	478.1 µL	690.6 µL	1328.1 µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1062.5 µL
100 mM dNTP Mix (green cap)	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	27.6 µL	53.1 µL
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	106.3 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	106.3 µL
Total Volume	**25 µL**	**318.9 µL**	**531.3 µL**	**743.9 µL**	**956.3 µL**	**1381.3 µL**	**2656.3 µL**

7 Using a fresh Nunc DeepWell plate, prepare the master mix source plate by adding the volume of PCR master mix indicated in **Table 72** to all wells of column 1 of the DeepWell plate.

Table 72 Preparation of the Master Mix Source Plate for Post-CapPCR_XT_LI_ILM protocol

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Post-Capture PCR Master Mix	Column 1 (A1-H1)	36.0 µL	62.0 µL	88.0 µL	114.0 µL	166.0 µL	322.0 µL

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

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

Load the Agilent NGS Workstation

10 Load the Labware MiniHub according to **Table 73**, using the plate orientations shown in **Figure 4** on page 41.

Table 73 Initial MiniHub configuration for Post-CapPCR_XT_LI_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

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

Table 74 Initial Bravo deck configuration for Post-CapPCR_XT_LI_ILM protocol

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

12 Load the BenchCel Microplate Handling Workstation according to **Table 75**.

Table 75 Initial BenchCel configuration for Post-CapPCR_XT_LI_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	—	—	—

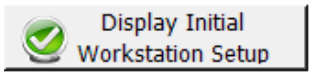
Run VWorks protocol Post-CapPCR_XT_LI_ILM

13 On the SureSelect setup form, under **Select Protocol to Run**, select the **Post-CapPCR_XT_LI_ILM** protocol.

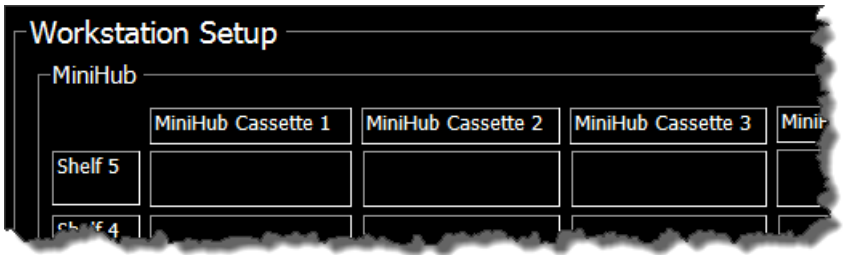
14 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

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

16 Click **Display Initial Workstation Setup**.



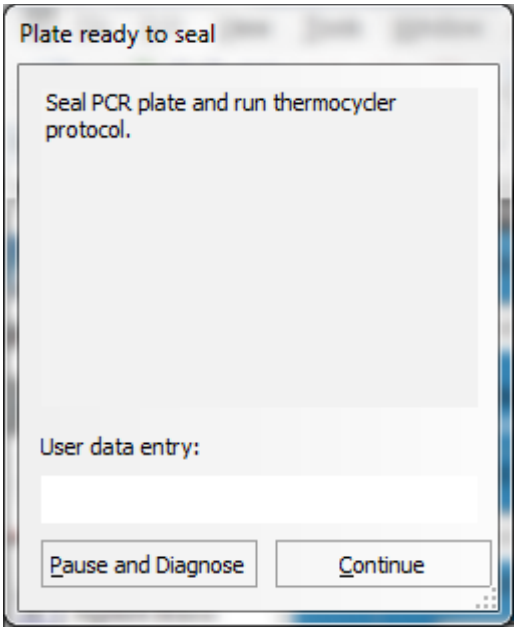
17 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

Running the Post-CapPCR_XT_LI_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.

19 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 3.0 seconds.



20 Place the plate in the thermal cycler. Resume the thermal cycling program in **Table 69** on page 91.

21 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.

Prepare the workstation and reagents

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

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

3 Pre-set the temperature of Bravo deck position 4 to 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 Verify that the AMPure XP bead suspension is at room temperature. *Do not freeze the beads at any time*.

6 Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.

7 Prepare a Nunc DeepWell source plate for the beads by adding 55 µL of homogeneous AMPure XP beads per well, for each well to be processed.

8 Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water.

9 Prepare a separate Thermo Scientific reservoir containing 45 mL of freshly-prepared 70% ethanol.

10 Load the Labware MiniHub according to **Table 76**, using the plate orientations shown in **Figure 4** on page 41.

Table 76 Initial MiniHub configuration for AMPureXP_XT_LI_ILM (Post-Capture PCR) protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	Empty Eppendorf twin.tec Plate	—	—
Shelf 2	—	Nuclease-free water reservoir from **step 8**	AMPure XP beads in Nunc DeepWell plate from **step 7**	—
Shelf 1 (Bottom)	—	70% ethanol reservoir from **step 9**	—	Empty tip box

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

Table 77 Initial Bravo deck configuration for AMPureXP_XT_LI_ILM (Post-Capture PCR) protocol

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
9	Amplified DNA libraries in unsealed PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)

12 Load the BenchCel Microplate Handling Workstation according to **Table 78**.

Table 78 Initial BenchCel configuration for AMPureXP_XT_LI_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	6 Tip boxes	—	—	—

Run VWorks protocol *AMPureXP_XT_LI_ILM* (Post-Capture PCR) protocol

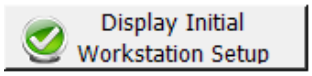
13 On the SureSelect setup form, under **Select Protocol to Run**, select the **AMPureXP_XT_LI_ILM** (Post-Capture PCR) protocol.

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.

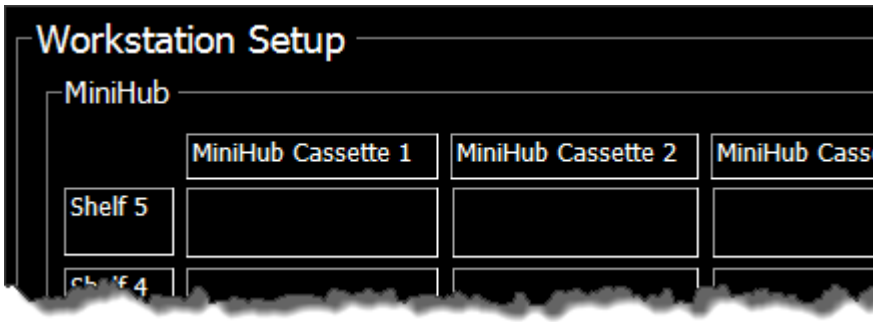
14 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate containing the amplified libraries at position 9.

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

16 Click **Display Initial Workstation Setup**.



17 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

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

Step 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 97.
- 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 102.

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 Eppendorf twin.tec plate that was used for the Hyb_XT_LI_ILM protocol run, prepare the Sample Buffer source plate at room temperature. Add the volume of High Sensitivity D1000 Sample Buffer indicated in **Table 79** to each well of column 4 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 13**.

Table 79 Preparation of the Sample Buffer Source Plate for TS_HighSensitivity_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Eppendorf twin.tec Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
High Sensitivity D1000 Sample Buffer	Column 4 (A4-H4)	8.0 µL	11.0 µL	14.0 µL	17.0 µL	23.0 µL	41.0 µL

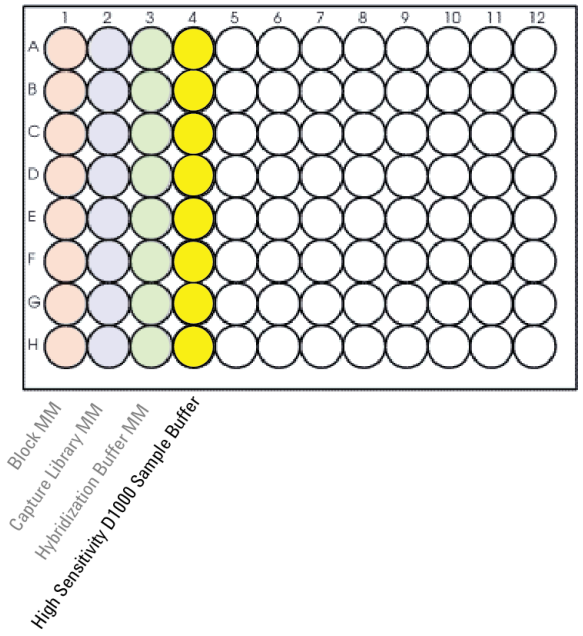


Figure 13 Configuration of the sample buffer source plate for TS_HighSensitivity_D1000 protocol. Columns 1-3 were used to dispense master mixes during the hybridization protocol.

Load the Agilent NGS Workstation

6 Load the Labware MiniHub according to **Table 80**, using the plate orientations shown in **Figure 4** on page 41.

Table 80 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 81**.

Table 81 Initial Bravo deck configuration for TS_HighSensitivity_D1000 protocol

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

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

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

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

Table 82 Initial BenchCel configuration for TS_HighSensitivity_D1000 protocol

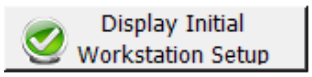
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Run VWorks protocol TS_HighSensitivity_D1000

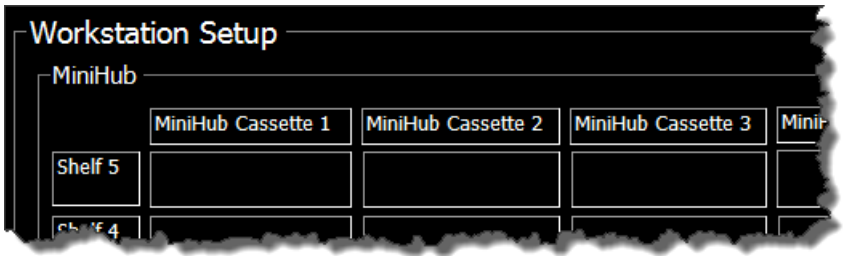
9 On the SureSelect setup form, under **Select Protocol to Run**, select the **TS_HighSensitivity_D1000** protocol.

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

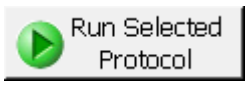
11 Click **Display Initial Workstation Setup**.



12 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.

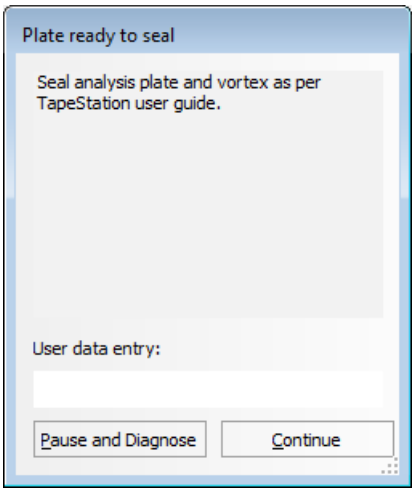


13 When verification is complete, click **Run Selected Protocol**.



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 103**.

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



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

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

Run the High Sensitivity D1000 Assay and analyze the data

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

16 Verify that the electropherogram shows the peak of DNA fragment size positioned between 200 and 400 bp. Sample electropherograms are shown in **Figure 14** (library prepared from high-quality DNA), **Figure 15** (library prepared from medium-quality FFPE DNA), and **Figure 16** (library prepared from low-quality FFPE DNA).

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

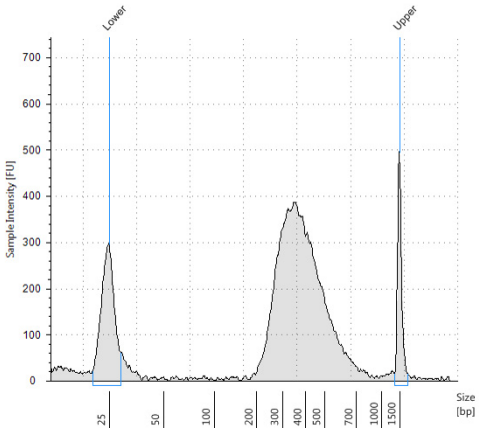


Figure 14 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

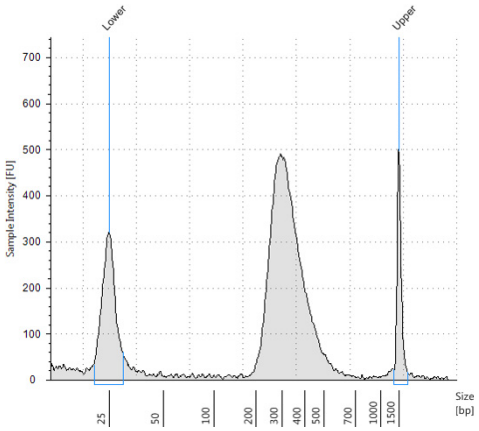


Figure 15 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

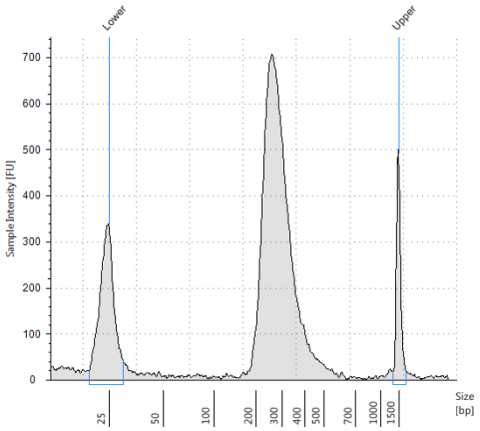


Figure 16 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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

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

Using manual preparation of the analytical samples, you can analyze the DNA samples on Agilent 2100 BioAnalyzer, Agilent 5200 Fragment Analyzer, or Agilent 4200/4150 TapeStation. Electropherograms obtained using one of these other analysis platforms are expected to show fragment size profiles similar to those shown for the Agilent 4200 TapeStation (see **Figure 14** through **Figure 16**). **Table 83** includes links to assay instructions.

Table 83 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

The number of indexed libraries (both single and dual indexed) 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 (for single indexed libraries) or index pairs (for dual indexed libraries) 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 following methods:

Method 1: Dilute each sample to be pooled to the same final concentration (typically 4 nM–15 nM, or the concentration of the most dilute sample) using Low TE, then combine equal volumes of all samples to create the final pool.

Method 2: Starting with samples at different concentrations, add the appropriate volume of each sample to achieve equimolar concentration in the pool, then adjust the pool to the desired final volume using Low TE. 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 84 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 84 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

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} Low Input 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 17**.

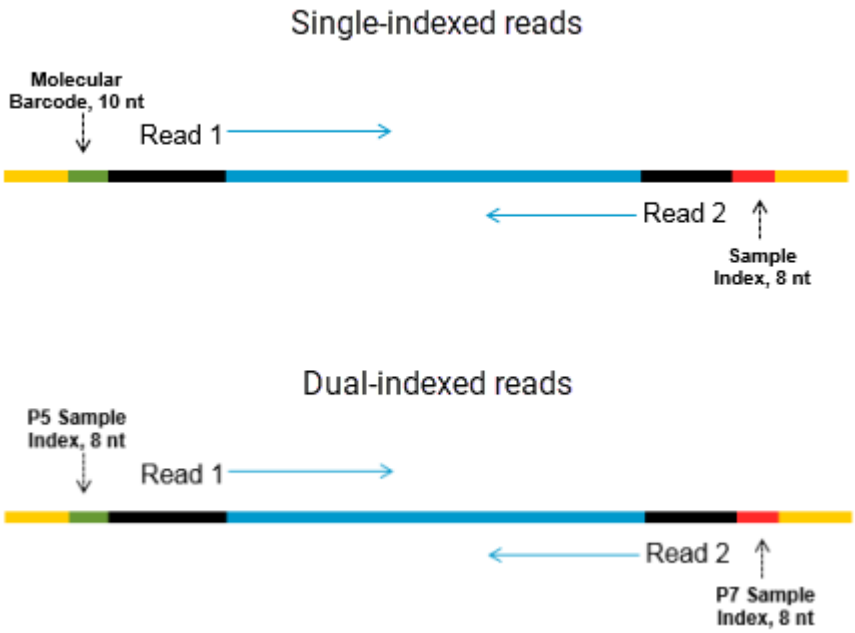


Figure 17 Content of SureSelect XT Low Input single-indexed sequencing library (top) and dual-indexed sequencing library (bottom). Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), and the library bridge PCR primers (yellow). In the single-indexed reads, the sample index is shown in red and the molecular barcode in green. In the dual-indexed reads, the P5 and P7 sample indexes are shown in green and red, respectively.

Libraries can be sequenced on the Illumina HiSeq, MiSeq, NextSeq, or NovaSeq platform using the run type and chemistry combinations shown in **Table 85**.

CAUTION

Do not use the HiSeq2500 instrument in high-output run mode (v4 chemistry) if your analysis pipeline includes molecular barcode (i5) reads. Poor molecular barcode sequence data quality (lower Q scores, with impacts on coverage and sensitivity of variant calls) has been observed when SureSelect^{XT} Low Input libraries are sequenced on the HiSeq 2500 instrument in this mode. Sequencing performance for Read 1, Read 2, and sample-level index (i7) reads is not affected and this platform/run mode/chemistry may be used for applications that omit molecular barcode analysis.

Similarly, for dual-indexed libraries, reduced P5 sample index quality has been observed when SureSelect^{XT} Low Input libraries are sequenced on the HiSeq 2500 instrument in high-output run mode.

See **Table 85** for alternative run mode/chemistry options for the HiSeq2500 platform.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See **Table 85** for kit configurations compatible with the recommended read length.

The optimal seeding concentration for SureSelect^{XT} Low Input target-enriched libraries varies according to sequencing platform, run type, and Illumina kit version. See **Table 85** 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 85**.

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

Table 85 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

* Review the *Caution* statement on **page 104** for important considerations regarding the use of the HiSeq 2500 platform in high-run mode.

Use the following additional considerations for sequencing set up for SureSelect Cancer All-In-One assays:

- For All-In-One assays that include translocation detection, Agilent strongly recommends using paired-end sequencing read length of at least 2 × 100 bp and preferably 2 × 150 bp.
- The *All-In-One Analysis* workflow in Agilent's SureCall application (v4.2) does not currently use molecular barcodes to remove duplicate reads. Collection of the 10-bp i5 index reads is therefore not required specifically for samples analyzed using this workflow.

Step 6. Do the sequencing run and analyze the data

Use the guidelines below for SureSelect^{XT} Low Input library sequencing run setup and analysis.

- The sample-level index (i7) requires an 8-bp index read. For complete i7 index sequence information, see **Table 100** and **Table 101** on page 127.

CAUTION

The 8-bp index sequences in SureSelect XT Low Input Index Primers 1-96 and 97-192 differ from the 8-bp index sequences in index primers A01 through H12 in Agilent's SureSelect XT system.

- For single-indexed libraries, the degenerate molecular barcode requires a 10-bp index read. For dual-indexed libraries, the P5 index (i5) requires an 8-bp index read.

NOTE

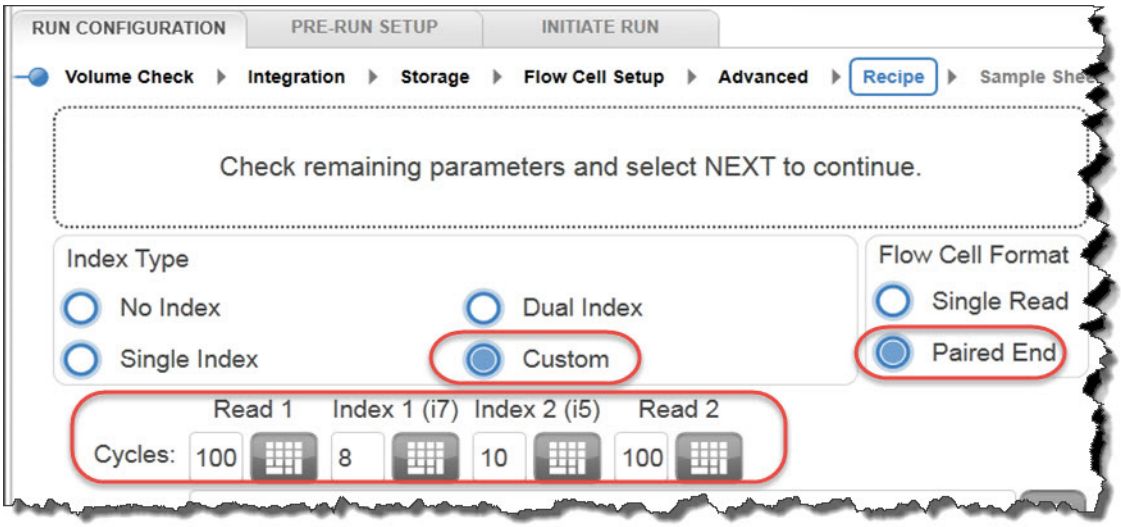
If molecular barcode analysis is not needed for single-indexed samples, you can modify the steps in this section to omit sequencing and analysis of i5 index reads. Use of the molecular barcodes is recommended for detection of very low allele frequency variants and when the DNA sample is present in limited amounts.

Note that if you are using the SureCall (v4.2) *All-In-One Analysis* workflow for analysis of SureSelect Cancer All-In-One assays, this workflow does not currently include molecular barcode analysis.

- For the HiSeq, NextSeq, and NovaSeq platforms, set up the run using the instrument's user interface, following the guidelines on **page 107** (single-indexed libraries) or **page 108** (dual-indexed libraries).
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed in **"Single-Indexed libraries: MiSeq platform sequencing run setup guidelines"** on page 109, or in **"Dual-Indexed libraries: MiSeq platform sequencing run setup guidelines"** on page 111.
- Do not use Illumina's IEM adaptor trimming options. Make sure any IEM adaptor trimming option checkboxes are cleared (deselected) when setting up the sequencing run. Adaptors are trimmed in later processing steps using the Agilent software tools described below to ensure proper processing.
- For single-indexed libraries, demultiplex using Illumina's bcl2fastq software, using the I2 MBC retrieval steps described below. See **page 107** for HiSeq, NextSeq, and NovaSeq runs and see **page 111** for MiSeq runs.
- For dual-indexed libraries, demultiplex using Illumina's bcl2fastq, BCL Convert or DRAGEN software to generate paired end reads based on the dual indexes and remove sequences with incorrectly paired P5 and P7 indexes.
- For human germline DNA variant analysis, you can use Agilent's Alissa Reporter software for the complete FASTQ file to variant discovery process (see **page 113** for more information).
- For germline or somatic variant analysis, you can use Agilent's AGeNT software modules to process the library read FASTQ files to analysis-ready BAM files (see **page 114** for more information).

Single-Indexed libraries: HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface. A sample run setup for the HiSeq platform using 100 + 100 bp paired-end sequencing is shown below.



If using the NextSeq or NovaSeq platform, locate the same parameters on the *Run Setup* screen, and populate the **Read Length** fields using the **Cycles** settings shown in HiSeq platform example above. In the **Custom Primers** section of the NextSeq or NovaSeq platform *Run Setup* screen, clear (do not select) the check boxes for all primers (*Read 1, Read 2, Index 1* and *Index 2*).

BaseSpace currently does not support the sequencing of molecular barcodes as index reads. Set up NextSeq runs using the stand-alone mode.

Retrieve I2 FASTQ files containing molecular barcodes

Retrieval of I2 index files containing the molecular barcode (i5) index reads requires offline conversion of .bcl to fastq files using one of the two methods below.

Option 1: Use bcl2fastq software with base masking

To generate Index 2 fastq files containing the P5 molecular barcodes using the bcl2fastq software, follow Illumina's instructions for use of the software with the following modifications:

1. Use of a sample sheet is mandatory and not optional. Modify the sample sheet to include only the sample index and not the molecular barcode index by clearing the contents in the I5_Index_ID and index2 columns.
2. Set *mask-short-adapter-reads* to value of 0.
3. Use the following base mask: Y*, I8, Y10, Y* (where * should be replaced with the actual read length, with the value entered matching the read length value in the RunInfo.xml file).

CAUTION

When generating fastq files using Illumina's bcl2fastq software, make sure to clear the contents of the index2 column in the sample sheet as described above. Do not enter an N_{10} sequence to represent the degenerate molecular barcode; instead, simply leave the column cells cleared.

The bcl2fastq software does not treat the "N" character as a wildcard when found in sample sheet index sequences, and usage in this context will cause a mismatch for any sequence character other than "N".

Option 2: Use Broad Institute Picard tools

To generate Index 2 fastq files containing the P5 molecular barcodes using the Broad Institute Picard tools, complete the following steps:

1 Use tool ExtractIlluminaBarcodes to find the barcodes. A sample set of commands is shown below (commands used by your facility may vary).

```
nohup java -jar picard.jar ExtractIlluminaBarcodes
BASECALLS_DIR=<sequencing_run_directory>/Data/Intensities/BaseCalls/
OUTPUT_DIR=<barcode_output_dir_name> LANE=1 READ_STRUCTURE=<read_structure>
BARCODE_FILE=<barcode_file> METRICS_FILE=<metric_file_name> NUM_PROCESSORS=<n>
```

2 Use tool IlluminaBaseCallsToFastq to generate the fastq files based on output of step 1. A sample set of commands is shown below (commands used by your facility may vary).

```
nohup java -jar picard.jar IlluminaBasecallsToFastq
BASECALLS_DIR=<sequencing_run_directory>/Data/Intensities/BaseCalls/ LANE=1
BARCODES_DIR=<barcode_output_dir_name> READ_STRUCTURE=<read_structure>
FLOWCELL_BARCODE=<FCID> MACHINE_NAME=<machine_name> RUN_BARCODE=<run_number>
ADAPTERS_TO_CHECK=PAIRED_END

NUM_PROCESSORS=<n> READ_NAME_FORMAT=CASAVA_1_8 COMPRESS_OUTPUTS=true
MULTIPLEX_PARAMS=<multiplex_params_file> IGNORE_UNEXPECTED_BARCODES=true
TMP_DIR=<temp_directory_location>
```

Dual-Indexed libraries: HiSeq/NextSeq/NovaSeq platform sequencing run setup guidelines

Set up sequencing runs using the instrument control software interface, using the settings shown in **Table 86**. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface and enter the Cycles settings in **Table 86**.

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

Table 86 Run settings

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

Single-Indexed libraries: 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 Low Input indexes used for each sample. See **Table 100** on page 126 and **Table 101** on page 127 for nucleotide sequences of the SureSelect XT Low Input system indexes.

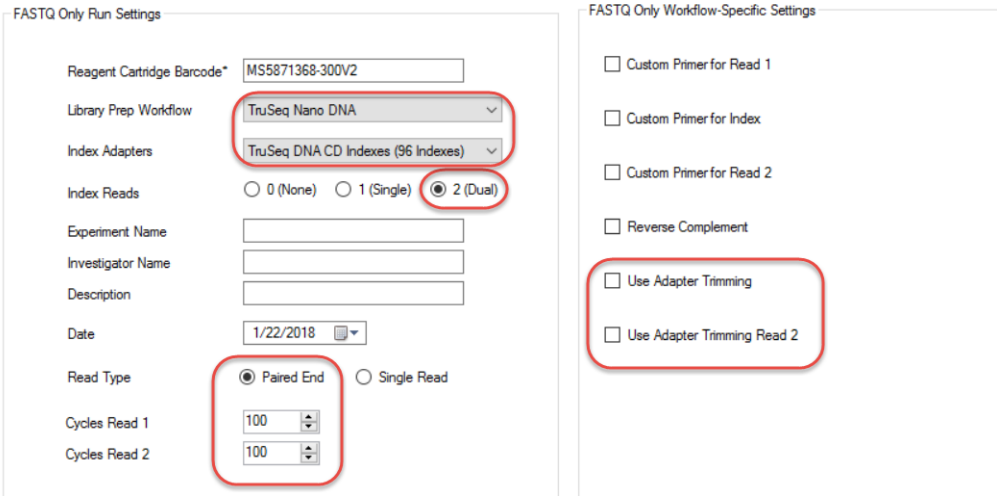
Set up a custom Sample Sheet:

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*.

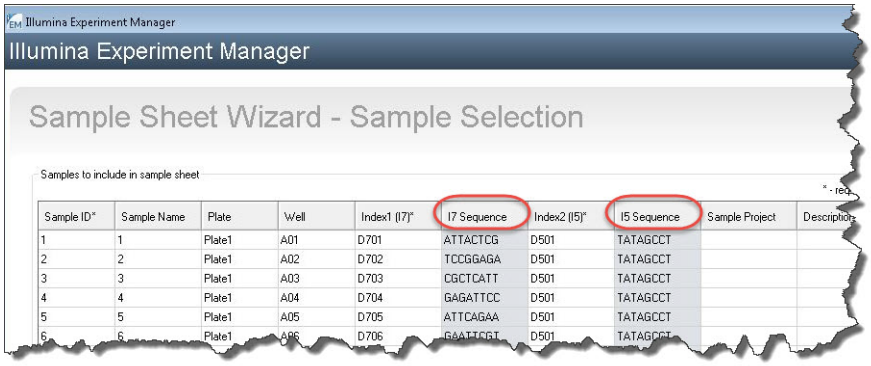
2 On the Workflow Parameters screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA**. In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. If your pipeline uses SureCall for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

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



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

Likewise, in the **I5 Sequence** column, assign any of the Illumina i5 indexes, to be corrected to the degenerate molecular barcode at a later stage.



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

Edit the Sample Sheet to include SureSelect XT Low Input indexes and molecular barcodes

1 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 in **Figure 18**).

- In column 5 under **I7_Index_ID**, enter the name of the SureSelect XT Low Input index assigned to the sample. In column 6 under index, enter the corresponding SureSelect XT Low Input Index sequence. See **Table 100** on page 126 and **Table 101** on page 127 for nucleotide sequences of the SureSelect XT Low Input indexes.
- In column 7 under **I5_Index_ID**, enter *MBC* for all samples. In column 8 under index2, enter text *NNNNNNNNNN* for all samples to represent the degenerate 10-nucleotide molecular barcode tagging each fragment.

NOTE

Enter N_{10} text in the index2 column only when sample sheets are processed using MiSeq Reporter software adjusted to retrieve I2 fastq files containing molecular barcodes, as detailed on **page 111**. Sample sheets processed offline using Illumina's bcl2fastq software must not contain N_{10} wildcard index sequences. See **page 108** for more information.

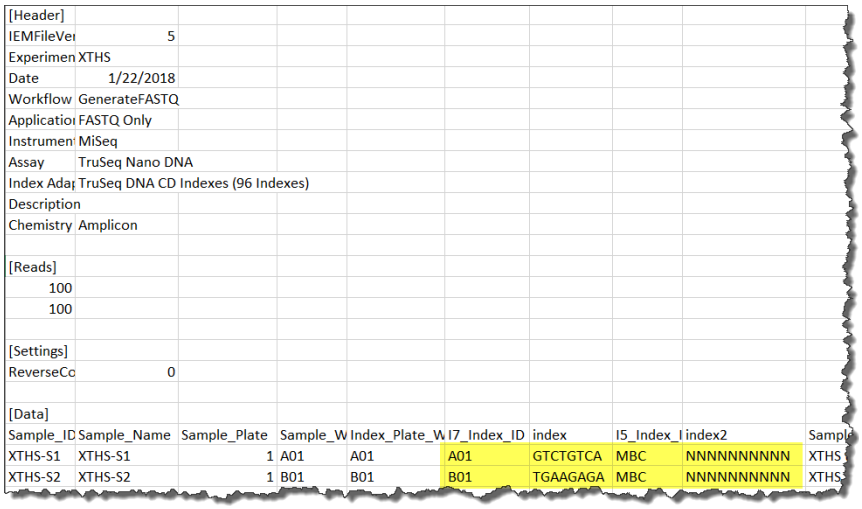


Figure 18 Sample sheet for use with MiSeq platform after MiSeq Reporter reconfiguration

2 Save the edited Sample Sheet in an appropriate file location for use in the MiSeq platform run.

Reconfigure the MiSeq Reporter Software to retrieve I2 FASTQ files

By default, MiSeq Reporter software does not generate fastq files for index reads. To generate fastq I2 index files containing the molecular barcode reads using MiSeq Reporter, adjust the software settings as described below before the first use of the MiSeq instrument for SureSelect XT Low Input library sequencing. Once changed, this setting is retained for future runs.

To change this setting, open the file **MiSeq Reporter.exe.config**. Under the **<appSettings>** tag, add **<add key="CreateFastqForIndexReads" value="1"/>**. You must restart the instrument for this setting change to take effect.

NOTE

If you are using the same instrument for assays other than SureSelect XT Low Input library sequencing, the configuration file should be edited to **<add key="CreateFastqForIndexReads" value="0"/>** and the instrument should be restarted before running the other assay.

If you are using the MiSeqDx platform, run the instrument in research mode to make changes to MiSeq Reporter settings. If research mode is not available on your instrument, you may need to upgrade the system to include the dual boot configuration to allow settings changes in research mode.

The alternative methods for retrieval of I2 fastq files described on **page 107** for HiSeq and NextSeq platform runs may also be applied to MiSeq platform runs.

Dual-Indexed libraries: MiSeq platform sequencing run setup guidelines

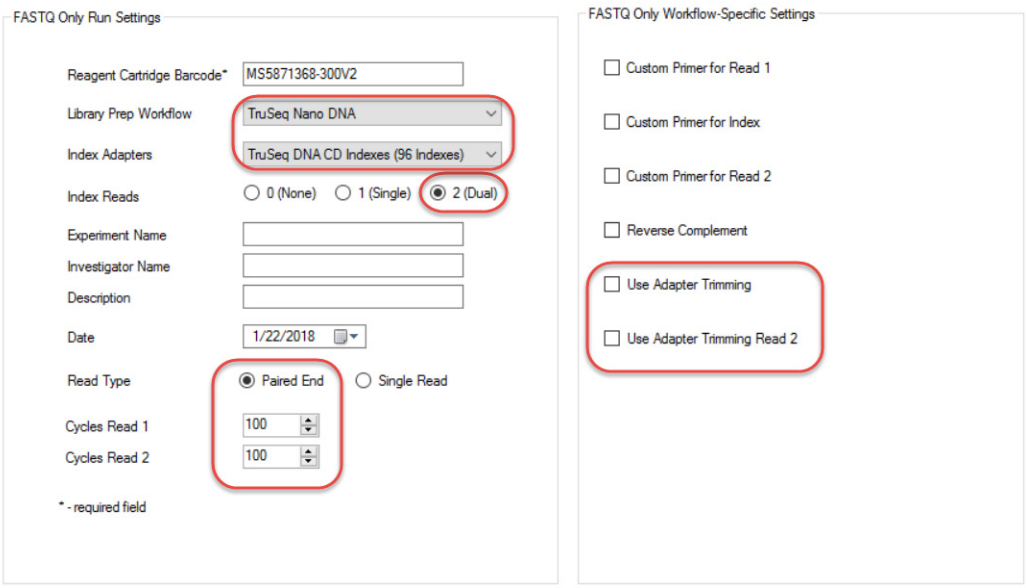
Use the Illumina Experiment Manager (IEM) software to generate a custom Sample Sheet according to the guidelines below. Once a Sample Sheet has been generated, index sequences need to be manually changed to the dual indexes used for each sample. See **Table 102** on page 128 for nucleotide sequences of the dual indexes.

Setting 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*.

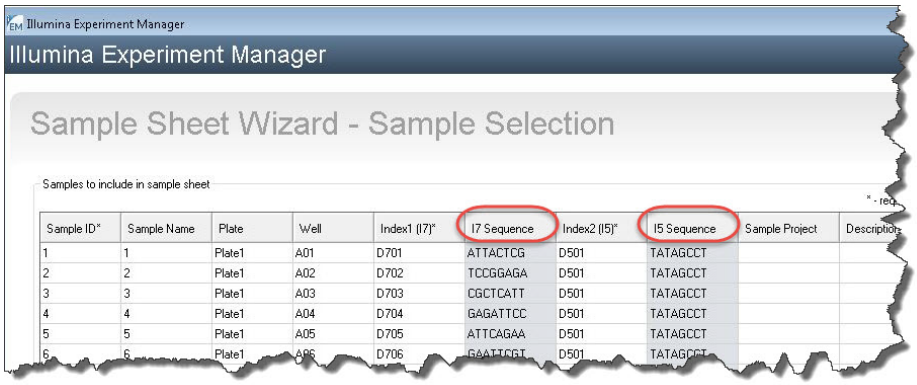
2 On the Workflow Parameters screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA**. In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. If your pipeline uses SureCall for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

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



3 Using the Sample Sheet Wizard, set up a New Plate, entering the required information for each sample to be sequenced. In the **I7 Sequence** column, assign each sample to any of the Illumina i7 indexes. The index will be corrected to a SureSelect XT Low Input index 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 Low Input Dual Index P5 Indexed Adaptor at a later stage.

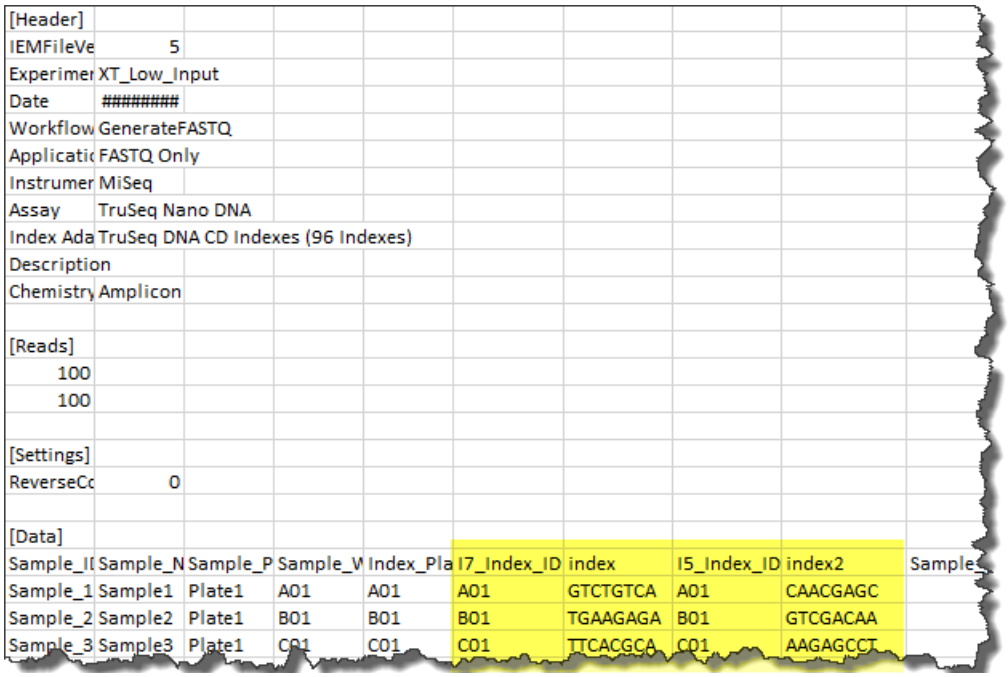


4 Finish the sample sheet setup tasks and save the sample sheet file.

Editing the Sample Sheet to include SureSelect XT Low Input dual indexes

1 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).

- In column 5 under **I7_Index_ID**, enter the name of the SureSelect XT Low Input index assigned to the sample. In column 6 under **index**, enter the corresponding P7 index sequence. See **Table 102** on page 128 for nucleotide sequences of the SureSelect XT Low Input indexes.
- In column 7 under **I5_Index_ID**, enter the name of the SureSelect XT Low Input Dual Index P5 Indexed Adaptor assigned to the sample. In column 8 under **index2**, enter the corresponding P5 index sequence. See **Table 102** on page 128 for nucleotide sequences of the index segment of the SureSelect XT Low Input Dual Index P5 Indexed Adaptors.



[Header]									
IEMFileVe	5								
Experimer	XT_Low_Input								
Date	########								
Workflow	GenerateFASTQ								
Applicatic	FASTQ Only								
Instrumer	MiSeq								
Assay	TruSeq Nano DNA								
Index Ada	TruSeq DNA CD Indexes (96 Indexes)								
Description									
Chemistry	Amplicon								
[Reads]									
100									
100									
[Settings]									
ReverseCc	0								
[Data]									
Sample_I	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 19 Sample sheet for SureSelect XT Low Input dual indexed library sequencing

2 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 Low Input DNA library data analysis. Your NGS analysis pipeline may vary. For SureSelect Cancer All- In- One assay sequence analysis guidelines, see the assay **Product Overview Guide**.

Prior to analysis, use the appropriate Illumina demultiplexing software to generate paired-end reads (see **page 106** for guidelines). The demultiplexed FASTQ data needs to be pre-processed to remove sequencing adaptors and utilize the i5 MBC reads (if collected) using one of the tools described below.

Using Agilent's Alissa Reporter software for germline DNA workflows

Alissa Reporter software provides a complete FASTQ-to-Result solution for Agilent's SureSelect assays, processing NGS data from FASTQ format to VCF format, and reporting human germline SNV, InDel and CNV calls.

Alissa Reporter is a cloud-based, multi-tenant software as a service (SaaS) product, delivering integrated pre- processing of SureSelect XT Low Input DNA library reads (adaptor trimming, MBC extraction and deduplication) along with secondary data analysis and quality control (QC) analytics using a built-in dashboard. To obtain more information and to purchase access to the software please visit the **Alissa Reporter page at www.agilent.com**.

Key considerations for SureSelect XT Low Input DNA assay steps prior to Alissa Reporter software analysis are summarized below:

- Alissa Reporter applications are available for germline analysis of human DNA libraries enriched using a pre-designed or custom SureSelect human probe (see **page 12**). Libraries enriched using SureSelect XT HS Human All Exon V7 or V8 probes are analyzed with the corresponding *Human All Exon V7 Germline* or *Human All Exon V8 Germline* application in Alissa Reporter. Libraries enriched using other probes, including additional pre-designed probes, are analyzed using an Alissa Reporter *Custom* application. The Alissa Reporter console provides tools for importing both pre-designed and custom probe designs from SureDesign and setting up a new *Custom* application for each imported design.

NOTE Human All Exon V8+UTR and Human All Exon V8+NCV designs must be imported into Alissa Reporter for use as *Custom*-type applications. Use the *Catalog*-type Alissa Reporter applications, including the *Human All Exon V8 Germline* application, only for the specific probe indicated for the application without any additional design content.

- Analysis of FFPE-derived or other DNA samples for detection of somatic variants is not supported at the time of this publication. Please visit the **Alissa Reporter page at www.agilent.com** for information on the latest Alissa Reporter software version capabilities.
- For CNV calling a co-analysis strategy is used in which unrelated samples from the same Alissa Reporter run are used to determine the reference signal for the target sample (no specific reference sample is required). At least 3 and preferably 8 (or more) unrelated samples need to be analyzed in Alissa Reporter together to obtain a reliable reference signal for CNV calls. For CNV calling on the X and Y chromosomes, unrelated samples of the same gender are required. For best results, process the samples to be used for CNV co-analysis in the same SureSelect run and in the same sequencing run in order to minimize any processing-based variance.
- Maximum file size for uploads is 50GB/file (in total 400GB/sample). A maximum of 768 FASTQ files can be uploaded in a run.
- File sizes >150M reads are randomly subsampled to 150M reads when using the *Human All Exon V7 Germline* or *Human All Exon V8 Germline* application.
- Unmerged and merged FASTQ files are supported. Upload of BAM files or other non-FASTQ file formats is not supported.

Using Agilent's AGeNT software for germline or somatic DNA workflows

Agilent's AGeNT software is a Java-based toolkit used for SureSelect XT Low Input DNA library read processing steps. The AGeNT tools are designed to enable building, integrating, maintaining, and troubleshooting internal analysis pipelines for users with bioinformatics expertise. For additional information and to download this toolkit, visit the **AGeNT page at www.agilent.com** and review **AGeNT Best Practices** for processing steps suitable for XT HS/Low Input DNA libraries, summarized below.

Prior to variant discovery, the AGeNT Trimmer module is used to pre-process the demultiplexed SureSelect XT Low Input library FASTQ data to trim sequencing adaptors and prepare MBC reads for insertion in the aligned BAM file.

The trimmed reads should be aligned, and MBC tags added to the aligned BAM file using a suitable tool such as BWA-MEM. Once alignment and tagging are complete, the AGeNT CReaK (Consensus Read Kit) tool is used to generate consensus reads and mark or remove duplicates. The resulting BAM files are ready for downstream analysis including variant discovery.

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

6 Appendix: Using FFPE-derived DNA Samples

This chapter summarizes the protocol modifications to apply to FFPE samples based on the integrity of the FFPE sample DNA.

Protocol modifications for FFPE Samples

Protocol modifications that should be applied to FFPE samples are summarized in **Table 87**.

Table 87 Summary of protocol modifications for FFPE samples

Workflow Step and page	Parameter	Condition for non-FFPE Samples	Condition for FFPE Samples
gDNA Sample Preparation **page 36**	Qualification of DNA Integrity	Not required	Required
Enzymatic fragmentation duration **page 39**	Duration of the 37°C fragmentation step	10–15 minutes, depending on read length requirements	15 minutes
DNA input for Library Preparation **page 37**	Input amount and means of quantification	10 ng to 200 ng, quantified by Qubit assay	Based on determined DNA integrity (see **Table 12** on page 37 and **Table 13** on page 37)
DNA Shearing **page 47**	Mode of DNA Shearing	2 × 120 seconds	240 seconds (continuous)
Pre-capture PCR **page 56**	Cycle number	8–11	11–14
Sequencing **page 120**	Output augmentation	Per project requirements	1× to 10× based on determined DNA integrity (see **Table 88** and **Table 89** on page 120)

Methods for FFPE Sample Qualification

DNA integrity may be assessed using the Agilent NGS FFPE QC Kit or using the Agilent 4200 TapeStation system and Genomic DNA ScreenTape.

The Agilent NGS FFPE QC Kit provides a qPCR-based assay for DNA sample integrity determination. Results include the precise quantity of amplifiable DNA in the sample to allow direct normalization of input DNA amount and a ΔΔCq DNA integrity score used to design other protocol modifications.

The Agilent 4200 TapeStation system, combined with the Genomic DNA ScreenTape assay, provides a electrophoresis-based method for determination of a DNA Integrity Number (DIN) score used to estimate amount of input DNA required for sample normalization and to design other protocol modifications.

Sequencing Output Recommendations for FFPE Samples

After determining the amount of sequencing output required for intact DNA samples to meet the goals of your project, use the guidelines below to determine the amount of extra sequencing output required for FFPE DNA samples.

Samples qualified using ΔΔCq: For samples qualified based on the ΔΔCq DNA integrity score, use the guidelines in **Table 88**. For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with ΔΔCq score of 1 requires 200–400 Mb of sequencing output to achieve the same coverage.

Table 88 Recommended sequencing augmentation for FFPE-derived DNA samples

ΔΔCq value	Recommended fold increase for FFPE-derived sample
<0.5	No extra sequencing output
between 0.5 and 2	Increase sequencing allocation by 2× to 4×
>2	Increase sequencing allocation by 5× to 10× or more

Samples qualified using DIN: For samples qualified based on the Genomic DNA ScreenTape assay DIN integrity score, use the guidelines in **Table 89**. For example, if your workflow demands 100 Mb output for intact DNA samples to achieve the required coverage, an FFPE sample with DIN score of 4 requires approximately 200–400 Mb of sequencing output to achieve the same coverage.

Table 89 Recommended sequencing augmentation for FFPE-derived DNA samples

DIN value	Recommended fold increase for FFPE-derived sample
≥8	No extra sequencing output
between 3 and 8	Increase sequencing allocation by 2× to 4×
<3	Increase sequencing allocation by 5× to 10× or more

7 Reference

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

Kit Contents

SureSelect XT Low Input Reagent Kits include the component kits listed in **Table 90** or **Table 91**. Detailed contents of each of the multi-part component kits are shown in **Table 92** through **Table 95**.

Table 90 Contents of SureSelect XT Low Input Reagent Kit p/n G9703A (96 Reactions)

Component Kit Name	Storage Condition	Component Kit p/n
SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0140
SureSelect XT Low Input Index Primers 1–96 for ILM (Pre PCR)	–20°C	5190-6444
SureSelect Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR)	Room Temperature	5190-9687
SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	–20°C	5190-9686

Table 91 Contents of SureSelect XT Low Input Reagent Kit p/n G9703B (96 Reactions)

Component Kit Name	Storage Condition	Component Kit p/n
SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR)	–20°C	5500-0140
SureSelect XT Low Input Index Primers 97–192 for ILM (Pre PCR)	–20°C	5190-6445
SureSelect Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR)	Room Temperature	5190-9687
SureSelect XT HS and XT Low Input Target Enrichment Kit ILM Hyb Module, Box 2 (Post PCR)	–20°C	5190-9686

Table 92 SureSelect XT HS and XT Low Input Library Preparation Kit (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
Adaptor Oligo Mix	tube with white cap
Forward Primer	tube with brown cap
100 mM dNTP Mix (25 mM each dNTP)	tube with green cap
Herculase II Fusion DNA Polymerase	tube with red cap
5× Herculase II Reaction Buffer	tube with clear cap

Table 93 SureSelect XT Low Input Index Primers for ILM Kits (Pre PCR) Content

Kit Component	Index Primers 1-96 (p/n 5190-6444)	Index Primers 97-192 (p/n 5190-6445)
SureSelect XT Low Input Index Primers for ILM (reverse primers containing 8-bp index sequence)	Index Primers 1 through 96, provided in yellow plate (Index Plate 1)*	Index Primers 97 through 192, provided in red plate (Index Plate 2)†

* See **Table 98** on page 125 for a plate map and see **Table 100** on page 126 for index sequences.

† See **Table 99** on page 125 for a plate map and see **Table 101** on page 127 for index sequences.

CAUTION The SureSelect XT Low Input Index Primers are provided in single-use aliquots. To avoid cross-contamination of libraries, use each well for a single library preparation reaction. Do not re-use any residual volume for subsequent experiments.

Table 94 SureSelect Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR) Content

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

Table 95 SureSelect XT HS and XT Low Input Target Enrichment Kit, ILM Hyb Module Box 2 (Post PCR) Content

Kit Component	Format
SureSelect Fast Hybridization Buffer	bottle
SureSelect XT HS and XT Low Input Blocker Mix	tube with blue cap
SureSelect RNase Block	tube with purple cap
SureSelect Post-Capture Primer Mix	tube with clear cap
100 mM dNTP Mix (25 mM each dNTP)	tube with green cap
Herculase II Fusion DNA Polymerase	tube with red cap
5× Herculase II Reaction Buffer	tube with clear cap

Bundles of SureSelect XT Low Input Reagent Kits with certain Target Enrichment Probes are available for purchase using the Agilent part numbers listed in **Table 96**. The SureSelect XT Low Input Reagent Kits included in these bundles are supplied with the same component kits listed in **Table 90** or **Table 91** on **page 122**.

Table 96 Supported SureSelect XT Low Input Reagent Kit + Probe Bundles for automation

Included SureSelect (SSel) XT Low Input Probe Capture Library	Included SureSelect XT Low Input Reagent Kit	
	96 Reactions, with Index Primers 1–96	96 Reactions, with Index Primers 97–192
Custom 1–499 kb*	G9507A	G9508A
Custom 0.5 –2.9 Mb*	G9507B	G9508B
Custom 3–5.9 Mb*	G9507C	G9508C
Custom 6–11.9 Mb*	G9507D	G9508D
Custom 12–24 Mb*	G9507E	G9508E
ClearSeq Comp Cancer	G9507G	G9508G
Clinical Research Exome V2	G9507H	G9508H
Clinical Research Exome V2 Plus	G9507J	G9508J
Human All Exon V6	G9507K	G9508K
Human All Exon V6 Plus	G9507L	G9508L
Human All Exon V6+UTRs	G9507M	G9508M
Human All Exon V7	G9507N	G9508N
Human All Exon V7 Plus 1	G9507P	G9508P
Human All Exon V7 Plus 2	G9507Q	G9508Q
Cancer All-In-One Lung	G9507R	G9508R
Cancer All-In-One Solid Tumor	G9507S	G9508S

* Kits that include Custom SureSelect Cancer All-In-One panels, designed using Agilent's SureDesign application, are ordered using these bundled custom design Agilent part numbers. The Custom SureSelect Cancer All-In-One panels are designated using design IDs beginning with an 'A' character. (Refer to the probe vial label and the associated Certificate of Analysis to view the design ID.)

The optional SureSelect Enzymatic Fragmentation Kit, 96 Reactions Automation (part number 5191-6764) includes the components shown in **Table 97**.

Table 97 SureSelect Enzymatic Fragmentation Kit Content

Kit Component	Format
5X SureSelect Fragmentation Buffer	tube with blue cap
SureSelect Fragmentation Enzyme	tube with green cap

P7 Index Reference Information

Table 98 Plate map for SureSelect XT Low Input P7 Index Primers 1-96, provided in yellow plate (Index Plate 1)

	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 99 Plate map for SureSelect XT Low Input P7 Index Primers 97-192, provided in red plate (Index Plate 2)

	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

The nucleotide sequence of the 8-nucleotide index portion of each indexing primer is provided in **Table 100** and **Table 101**. See **page 106** for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 100 SureSelect XT Low Input Indexes 1–96, provided in yellow 96-well plate (Index Plate 1)

Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence
1	A01	GTCTGTCA	25	A04	CCGTGAGA	49	A07	ATGCCTAA	73	A10	ACAGCAGA
2	B01	TGAAGAGA	26	B04	GACTAGTA	50	B07	ATCATTCC	74	B10	AAGAGATC
3	C01	TTCACGCA	27	C04	GATAGACA	51	C07	AACTCACC	75	C10	CAAGACTA
4	D01	AACGTGAT	28	D04	GCTCGGTA	52	D07	AACGCTTA	76	D10	AAGACGGA
5	E01	ACCACTGT	29	E04	GGTGCGAA	53	E07	CAGCGTTA	77	E10	GCCAAGAC
6	F01	ACCTCCAA	30	F04	AACAACCA	54	F07	CTCAATGA	78	F10	CTGTAGCC
7	G01	ATTGAGGA	31	G04	CGGATTGC	55	G07	AATGTTGC	79	G10	CGCTGATC
8	H01	ACACAGAA	32	H04	AGTCACTA	56	H07	CAAGGAGC	80	H10	CAACCACA
9	A02	GCGAGTAA	33	A05	AAACATCG	57	A08	GAATCTGA	81	A11	CCTCCTGA
10	B02	GTCGTAGA	34	B05	ACGTATCA	58	B08	GAGCTGAA	82	B11	TCTTCACA
11	C02	GTGTTCTA	35	C05	CCATCCTC	59	C08	GCCACATA	83	C11	GAACAGGC
12	D02	TATCAGCA	36	D05	GGAGAACA	60	D08	GCTAACGA	84	D11	ATTGGCTC
13	E02	TGGAACAA	37	E05	CGAACTTA	61	E08	GTACGCAA	85	E11	AAGGACAC
14	F02	TGGTGGTA	38	F05	ACAAGCTA	62	F08	TCCGTCTA	86	F11	ACACGACC
15	G02	ACTATGCA	39	G05	CTGAGCCA	63	G08	CAGATCTG	87	G11	ATAGCGAC
16	H02	CCTAATCC	40	H05	ACATTGGC	64	H08	AGTACAAG	88	H11	CCGAAGTA
17	A03	AGCAGGAA	41	A06	CATACCAA	65	A09	AGGCTAAC	89	A12	CCTCTATC
18	B03	AGCCATGC	42	B06	CAATGGAA	66	B09	CGACTGGA	90	B12	AACCGAGA
19	C03	TGGCTTCA	43	C06	ACGCTCGA	67	C09	CACCTTAC	91	C12	GATGAATC
20	D03	CATCAAGT	44	D06	CCAGTTCA	68	D09	CACTTCGA	92	D12	GACAGTGC
21	E03	CTAAGGTC	45	E06	TAGGATGA	69	E09	GAGTTAGC	93	E12	CCGACAAC
22	F03	AGTGGTCA	46	F06	CGCATACA	70	F09	CTGGCATA	94	F12	AGCACCTC
23	G03	AGATCGCA	47	G06	AGAGTCAA	71	G09	AAGGTACA	95	G12	ACAGATTC
24	H03	ATCCTGTA	48	H06	AGATGTAC	72	H09	CGACACAC	96	H12	AATCCGTC

Table 101 SureSelect XT Low Input Indexes 97–192, provided in red 96-well plate (Index Plate 2)

Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence
97	A01	CCACACGA	121	A04	CTGTCAGT	145	A07	TCGAACGC	169	A10	GACCTCCT
98	B01	GACCACAC	122	B04	TCTAGTGT	146	B07	GCCTAAAT	170	B10	ACAAGGAC
99	C01	GTGCGACA	123	C04	GGATGATA	147	C07	CGTGATAA	171	C10	CCAAACCT
100	D01	GCTTCATG	124	D04	TACAGCGT	148	D07	TCCGCTGA	172	D10	CACCAGTT
101	E01	ACTAAGTC	125	E04	AGTACCGA	149	E07	GCTCATTG	173	E10	TGGACGAC
102	F01	CAGGAAAG	126	F04	GAGCCAAG	150	F07	AATCGATG	174	F10	GTTACAGC
103	G01	GATCCGCT	127	G04	AGCGACAT	151	G07	TTCCATCA	175	G10	GAACAATG
104	H01	GTATCAAC	128	H04	TTACCACC	152	H07	ATTCACAG	176	H10	CAATGACT
105	A02	TAGAGTCG	129	A05	AGACGCCA	153	A08	CGGAAAGA	177	A11	GCTCGAAC
106	B02	TCGACACT	130	B05	CATACTGG	154	B08	GTCAAGTG	178	B11	TCGGTAGC
107	C02	CTGACCTC	131	C05	CACGCATT	155	C08	CATCTTCA	179	C11	TACGAACT
108	D02	CATGGCTT	132	D05	TGGTCAAG	156	D08	GATAGGAT	180	D11	GCCGGATT
109	E02	GTACAGAT	133	E05	GACGGAAA	157	E08	CAAGTGGT	181	E11	TAGCTCGG
110	F02	TAGTGTTC	134	F05	AGTAGACT	158	F08	GCGTTACA	182	F11	TTGCCGGA
111	G02	ATCGAAAC	135	G05	TACAAAGG	159	G08	TATGCAAC	183	G11	GGTATGGT
112	H02	TCAAGTCA	136	H05	CGCAAGAT	160	H08	GAGACCGT	184	H11	TCACTAAG
113	A03	GGAACAAT	137	A06	TGTTGCAA	161	A09	TCGATGAA	185	A12	CCTCCCAT
114	B03	TAGCGAGT	138	B06	ATCAACGT	162	B09	TCAAAGAG	186	B12	GTTCTAGT
115	C03	TACCGAAG	139	C06	GACGACTG	163	C09	GTGGTATG	187	C12	GAGAAACC
116	D03	TAAGTCAC	140	D06	ACTGGACG	164	D09	CTGAGAAT	188	D12	CCTGTAAT
117	E03	ATAACGTG	141	E06	TGATAACG	165	E09	TCTATCCG	189	E12	CCTTACCA
118	F03	GGTAGCTC	142	F06	ACATAGCG	166	F09	GCAATGTT	190	F12	ATGATAGG
119	G03	GAAGTACC	143	G06	ACACAAGG	167	G09	CACATAGC	191	G12	TATGGTGG
120	H03	CAACGTAT	144	H06	GAACGCTC	168	H09	TCCTGACC	192	H12	TGAGGAAT

Dual Indexing Reference Information

The content of the SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM kit required for the dual indexing protocol is described in **Table 102** below.

Table 102 SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM p/n 5191-4056 Content

Kit Component	Storage Condition	Format
SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 for ILM	–20°C	P5 Indexed Adaptors 1 through 96 (adaptor oligos containing 8-bp P5 index sequence), provided in green plate*

* See **Table 103** for a plate map and see **Table 104** on page 129 or **Table 105** on page 130 for the P5 index sequences included in each adaptor.

Table 103 Plate map for SureSelect XT Low Input Dual Index P5 Indexed Adaptors 1-96 (green 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

CAUTION The SureSelect XT Low Input Dual Index P5 Indexed Adaptors are provided in single-use aliquots. To avoid cross-contamination of libraries, use each well for a single library preparation reaction. Do not re-use any residual volume for subsequent experiments.

Nucleotide Sequences of the P5 indexes For NGS using the HiSeq 2500 and MiSeq platforms or using the NovaSeq 6000 platform with v1.0 chemistry, refer to the P5 index sequences in **Table 104** below. For the HiSeq 3000/4000 and NextSeq platforms, or using the NovaSeq 6000 platform with v1.5 chemistry, refer to **Table 105** on page 130.

Table 104 P5 Indexes 1–96 (green plate) for HiSeq 2500, MiSeq, or NovaSeq 6000 (v1.0 chemistry)

Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence
1	A01	CAACGAGC	25	A04	CCTGCGTG	49	A07	CGATACAG	73	A10	ACTCAGGC
2	B01	GTCGACAA	26	B04	TTCCAACA	50	B07	CCGTACTC	74	B10	GACCGCAT
3	C01	AAGAGCCT	27	C04	AGGCCTAG	51	C07	GTCGGTGT	75	C10	AGAGAGAA
4	D01	ACACCTTA	28	D04	AACGTGTC	52	D07	CTGACTAA	76	D10	TCAGATTG
5	E01	TGATCGCG	29	E04	GAGCCGCT	53	E07	ACTGGATG	77	E10	AAGATAGC
6	F01	TGGCTAGA	30	F04	ACATTACG	54	F07	TTGGCATG	78	F10	CGATTGGT
7	G01	GCCTCCGA	31	G04	TGCGACAT	55	G07	GACTCGTT	79	G10	GTTCCAAG
8	H01	CAGCGTTG	32	H04	TGTCCGGC	56	H07	CTCCGAAC	80	H10	CAATCTCG
9	A02	GTGTCTCA	33	A05	CTGTTCGC	57	A08	AATGGCAT	81	A11	CAAGTCAA
10	B02	ATAACATC	34	B05	TCACGCGA	58	B08	TAGCTGTA	82	B11	CAGTCGTG
11	C02	AACTTCCT	35	C05	GTTGTTCT	59	C08	GCTCACAC	83	C11	TTACAGTG
12	D02	GCGTTGGT	36	D05	ATTAACCG	60	D08	CTAATGTT	84	D11	ACCGGCCT
13	E02	CTAGCAAC	37	E05	CGTAGTAA	61	E08	CGTTACGT	85	E11	TCATTCCA
14	F02	TCTCGATC	38	F05	CACGCTGT	62	F08	GCGCATCA	86	F11	GCTAGGAT
15	G02	GTATGCGC	39	G05	TGGAACAG	63	G08	CCATCTAA	87	G11	ATGAATTG
16	H02	AGGTCGTT	40	H05	GTGTCGGC	64	H08	CGTCTCTT	88	H11	TTAGGCTC
17	A03	GTCAATAG	41	A06	AGAGTTCG	65	A09	ACCTTGTT	89	A12	TAACACCA
18	B03	CCTGTGAC	42	B06	TAGAACGC	66	B09	TATCGACG	90	B12	ACACTCTT
19	C03	GAGGAATA	43	C06	GAGATTAT	67	C09	TTGGCGAC	91	C12	CTGTATGA
20	D03	TGCTATCT	44	D06	TAATGAGA	68	D09	CGGAAGAT	92	D12	TTGGTCAA
21	E03	GATATCAC	45	E06	CTTGCCAA	69	E09	CAAGTATT	93	E12	CGTTGGCA
22	F03	CCTGAAGA	46	F06	CGCACAGA	70	F09	TGACGACT	94	F12	TCCACTTG
23	G03	TCTCTCAA	47	G06	GCGACTGT	71	G09	CGGCCATA	95	G12	AACGGTCA
24	H03	TTCCGTCT	48	H06	AGAATAAC	72	H09	TAAGTGGT	96	H12	CTGGACCA

Table 105 P5 Indexes 1–96 (green plate) for NextSeq*, HiSeq 3000/4000 or NovaSeq 6000 (v1.5 chemistry)

Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence	Index	Well	Sequence
1	A01	GCTCGTTG	25	A04	CACGCAGG	49	A07	CTGTATCG	73	A10	GCCTGAGT
2	B01	TTGTCGAC	26	B04	TGTTGGAA	50	B07	GAGTACGG	74	B10	ATGCGGTC
3	C01	AGGCTCTT	27	C04	CTAGGCCT	51	C07	ACACCGAC	75	C10	TTCTCTCT
4	D01	TAAGGTGT	28	D04	GACACGTT	52	D07	TTAGTCAG	76	D10	CAATCTGA
5	E01	CGCGATCA	29	E04	AGCGGCTC	53	E07	CATCCAGT	77	E10	GCTATCTT
6	F01	TCTAGCCA	30	F04	CGTAATGT	54	F07	CATGCCAA	78	F10	ACCAATCG
7	G01	TCGGAGGC	31	G04	ATGTCGCA	55	G07	AACGAGTC	79	G10	CTTGGAAC
8	H01	CAACGCTG	32	H04	GCCGGACA	56	H07	GTTCGGAG	80	H10	CGAGATTG
9	A02	TGAGACAC	33	A05	GCGAACAG	57	A08	ATGCCATT	81	A11	TTGACTTG
10	B02	GATGTTAT	34	B05	TCGCGTGA	58	B08	TACAGCTA	82	B11	CACGACTG
11	C02	AGGAAGTT	35	C05	AGAACAAC	59	C08	GTGTGAGC	83	C11	CACTGTAA
12	D02	ACCAACGC	36	D05	CGGTTAAT	60	D08	AACATTAG	84	D11	AGGCCGGT
13	E02	GTTGCTAG	37	E05	TTACTACG	61	E08	ACGTAACG	85	E11	TGGAATGA
14	F02	GATCGAGA	38	F05	ACAGCGTG	62	F08	TGATGCGC	86	F11	ATCCTAGC
15	G02	GCGCATAC	39	G05	CTGTTCCA	63	G08	TTAGATGG	87	G11	CAATTCAT
16	H02	AACGACCT	40	H05	GCCGACAC	64	H08	AAGAGACG	88	H11	GAGCCTAA
17	A03	CTATTGAC	41	A06	CGAACTCT	65	A09	AACAAGGT	89	A12	TGGTGTTA
18	B03	GTCACAGG	42	B06	GCGTTCTA	66	B09	CGTCGATA	90	B12	AAGAGTGT
19	C03	TATTCCTC	43	C06	ATAATCTC	67	C09	GTCGCCAA	91	C12	TCATACAG
20	D03	AGATAGCA	44	D06	TCTCATTA	68	D09	ATCTTCCG	92	D12	TTGACCAA
21	E03	GTGATATC	45	E06	TTGGCAAG	69	E09	AATACTTG	93	E12	TGCCAACG
22	F03	TCTTCAGG	46	F06	TCTGTGCG	70	F09	AGTCGTCA	94	F12	CAAGTGGA
23	G03	TTGAGAGA	47	G06	ACAGTCGC	71	G09	TATGGCCG	95	G12	TGACCGTT
24	H03	AGACGGAA	48	H06	GTTATTCT	72	H09	ACCACTTA	96	H12	TGGTCCAG

* P5 Index sequences shown in this table are for the NextSeq platform without use of BaseSpace. If using BaseSpace, see the P5 Index sequences shown in **Table 104**.

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} Low Input Automated Target Enrichment System protocol.

Enzymatic Fragmentation

Table 106 Fragmentation master mix - used on page 39

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2 µL	34.0 µL	51.0 µL	68.0 µL	85.0 µL	114.8 µL	221.0 µL
5X SureSelect Fragmentation Buffer (blue cap)	2 µL	34.0 µL	51.0 µL	68.0 µL	85.0 µL	114.8 µL	221.0 µL
SureSelect Fragmentation Enzyme (green cap)	1 µL	17.0 µL	25.5 µL	34.0 µL	42.5 µL	57.4 µL	110.5 µL
Total Volume	**5 µL**	**85 µL**	**127.5 µL**	**170 µL**	**212.5 µL**	**287 µL**	**552.5 µL**

Table 107 Master Mix Source Plate for enzymatic fragmentation protocol EnzFrag_XT_LI_ILM - used on page 40

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
Fragmentation master mix	Column 1 (A1-H1)	10.0 µL	15.0 µL	20.0 µL	25.0 µL	35.0 µL	68.0 µL

Library Preparation

Table 108 End Repair/dA-Tailing master mix - used on page 49

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 (orange cap)	4 µL	51 µL	85µL	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 109 Ligation master mix - used on page 49

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 (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 110 Adaptor Oligo Mix dilution - used on page 50

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	266.5 µL
Adaptor Oligo Mix (white cap)	5 µL	85.0 µL	127.5 µL	170.0 µL	212.5 µL	287.0 µL	552.5 µL
Total Volume	**7.5 µL**	**127.5 µL**	**191.3 µL**	**255.0 µL**	**318.8 µL**	**430.5 µL**	**819 µL**

Table 111 Master Mix Source Plate for library preparation runset LibraryPrep_XT_LI_ILM - used on page 50

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
End Repair-dA Tailing master mix	Column 2 (A2-H2)	31.0 µL	52.0 µL	73.0 µL	94.0 µL	136.0 µL	283.0 µL
Ligation master mix	Column 3 (A3-H3)	36.0 µL	62.0 µL	88.0 µL	114.0 µL	166.0 µL	348.0 µL
Adaptor Oligo Mix dilution (Single Indexing runs only)	Column 4 (A4-H4)*	15.0 µL	22.5 µL	30.0 µL	37.5 µL	52.5 µL	101.3 µL

* Leave Column 4 empty when preparing the source plate for a Dual Indexing run. The P5 indexed adaptors that replace the Adaptor Oligo Mix in these runs are supplied in a separate source plate.

Pre-Capture PCR

Table 112 Pre-Capture PCR master mix - used on page 56

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	9.5 µL	121.1 µL	201.9 µL	282.6 µL	363.4 µL	524.9 µL	1051.7 µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1107 µL
100 mM dNTP Mix (green cap)	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	27.6 µL	55.4 µL
Forward Primer (brown cap)	2 µL	25.5 µL	42.5 µL	59.5 µL	76.5 µL	110.5 µL	221.0 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.7 µL
Total Volume	**23.0 µL**	**293.3 µL**	**488.8 µL**	**684.3 µL**	**879.8 µL**	**1270.8 µL**	**2545.8 µL**

Table 113 Master Mix Source Plate for pre-capture PCR protocol Pre-CapPCR_XT_LI_ILM - used on page 56

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Pre-Capture PCR Master Mix	Column 5 (A5-H5)	34.5 µL	57.5 µL	80.5 µL	103.5 µL	149.5 µL	310.5 µL

Hybridization

Table 114 Block 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
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 HS and XT Low Input 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 115 Hybridization Buffer 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
Nuclease-free water	2.5 µL	53.1 µL	74.4 µL	95.6 µL	116.9 µL	159.4 µL	297.5 µL
SureSelect Fast Hybridization Buffer (bottle)	6.0 µL	127.5 µL	178.5 µL	229.5 µL	280.5 µL	382.5 µL	714.0 µL
Total Volume	**8.5 µL**	**180.6 µL**	**252.9 µL**	**325.1 µL**	**397.4 µL**	**541.9 µL**	**1011.5 µL**

Table 116 Capture Library Master Mix for Probes <3 Mb, 8 rows of wells - used on page 75

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	76.5 µL	114.8 µL	153.0 µL	191.3 µL	306.0 µL	592.9 µL
RNase Block (purple cap)	0.5 µL	8.5 µL	12.8 µL	17.0 µL	21.3 µL	34.0 µL	65.9 µL
Probe Capture Library	2.0 µL	34.0 µL	51.0 µL	68.0 µL	85.0 µL	136.0 µL	263.5 µL
Total Volume	**7.0 µL**	**119.0 µL**	**178.6 µL**	**238.0 µL**	**297.6 µL**	**476.0 µL**	**922.3 µL**

Table 117 Capture Library Master Mix for Probes ≥3 Mb, 8 rows of wells - used on page 75

Target size ≥3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	25.5 µL	38.3 µL	51.0 µL	63.8 µL	102.0 µL	197.6 µL
RNase Block (purple cap)	0.5 µL	8.5 µL	12.8 µL	17.0 µL	21.3 µL	34.0 µL	65.9 µL
Probe Capture Library	5.0 µL	85.0 µL	127.5 µL	170.0 µL	212.5 µL	340.0 µL	658.8 µL
Total Volume	**7.0 µL**	**119.0 µL**	**178.6 µL**	**238.0 µL**	**297.6 µL**	**476.0 µL**	**922.3 µL**

Table 118 Capture Library Master Mix for Probes <3 Mb, single row of wells - used on page 76

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µL	9.0 µL	13.8 µL	18.6 µL	23.3 µL	37.7 µL	73.5 µL
RNase Block (purple cap)	0.5 µL	1.0 µL	1.5 µL	2.1 µL	2.6 µL	4.2 µL	8.2 µL
Probe Capture Library	2.0 µL	4.0 µL	6.1 µL	8.3 µL	10.4 µL	16.8 µL	32.7 µL
Total Volume	**7.0 µL**	**14.0 µL**	**21.4 µL**	**29.0 µL**	**36.3 µL**	**58.7 µL**	**114.4 µL**

Table 119 Capture Library Master Mix for Probes ≥3 Mb, single row of wells - used on page 76

Target size >3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µL	3.0 µL	4.6 µL	6.2 µL	7.8 µL	12.6 µL	24.5 µL
RNase Block (purple cap)	0.5 µL	1.0 µL	1.5 µL	2.1 µL	2.6 µL	4.2 µL	8.2 µL
Probe Capture Library	5.0 µL	10.0 µL	15.3 µL	20.6 µL	25.9 µL	41.9 µL	81.7 µL
Total Volume	**7.0 µL**	**14.0 µL**	**21.4 µL**	**28.9 µL**	**36.3 µL**	**58.7 µL**	**114.4 µL**

Table 120 Master Mix Source Plate for hybridization protocol Hyb_XT_LI_ILM - used on page 76

Master Mix Solution	**Position on Source Plate**	**Volume of Master Mix added per Well of Eppendorf twin.tec Source Plate**					
		1-Column Runs	**2-Column Runs**	**3-Column Runs**	**4-Column Runs**	**6-Column Runs**	**12-Column Runs**
Block master mix	Column 1 (A1-H1)	11.0 µL	19.0 µL	27.0 µL	34.9 µL	50.9 µL	102.7 µL
Capture Library master mix	Column 2 (A2-H2)	14.0 µL	21.4 µL	28.9 µL	36.3 µL	58.6 µL	114.4 µL
Hybridization Buffer master mix	Column 3 (A3-H3)	19.9 µL	29.0 µL	38.0 µL	47.0 µL	65.1 µL	123.8 µL

Hybrid Capture and Washing

Table 121 Magnetic bead washing mixture - used on page 83

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	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 122 Resuspension of magnetic beads - used on page 83

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 123 Post-Capture PCR master mix - used on page 91

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	12.5 µL	159.4 µL	265.6 µL	371.9 µL	478.1 µL	690.6 µL	1328.1 µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1062.5 µL
100 mM dNTP Mix (green cap)	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	27.6 µL	53.1 µL
SureSelect Post-Capture Primer Mix (clear cap)	1 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	106.3 µL
Herculase II Fusion DNA Polymerase (red cap)	1 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	106.3 µL
Total Volume	**25 µL**	**318.9 µL**	**531.3 µL**	**743.9 µL**	**956.3 µL**	**1381.3 µL**	**2656.3 µL**

Table 124 Master Mix Source Plate for post-capture PCR protocol Post-CapPCR_XT_LI_ILM - used on page 92

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Post-Capture PCR Master Mix	Column 1 (A1-H1)	36.0 µL	62.0 µL	88.0 µL	114.0 µL	166.0 µL	322.0 µL

Troubleshooting Guide

If recovery of gDNA from samples is low

- ✔ Using excess tissue for gDNA isolation can reduce yield. Use only the amount of each specific tissue type recommended by the gDNA isolation protocol.
- ✔ Tissue sample lysis may not have been optimal during gDNA isolation. Monitor the extent of sample lysis during the Proteinase K digestion at 56°C by gently pipetting the digestion reaction every 20–30 minutes, visually inspecting the solution for the presence of tissue clumps. If clumps are still present after the 1-hour incubation at 56°C, add another 10 µL of Proteinase K and continue incubating at 56°C, with periodic mixing and visual inspections, for up to two additional hours. When the sample no longer contains clumps of tissue, move the sample to room temperature until lysis is complete for the remaining samples. Do not over-digest. Individual samples may be kept at room temperature for up to 2 hours before resuming the protocol. Do not exceed 3 hours incubation at 56°C for any sample.

If yield of pre-capture libraries is low

- ✔ The library preparation protocol includes specific thawing, temperature control, pipetting, and mixing instructions which are required for optimal performance of the highly viscous buffer and enzyme solutions used in the protocol. Be sure to adhere to all instructions when setting up the reactions.
- ✔ PCR cycle number may require optimization. Repeat library preparation for the sample, increasing the pre-capture PCR cycle number by 1 to 2 cycles. If a high molecular weight peak (>500 bp) is observed in the electropherogram for a sample with low yield, the DNA may be overamplified. Repeat library preparation for the sample, decreasing the pre-capture PCR cycle number by 1 to 3 cycles.
- ✔ DNA isolated from degraded samples, including FFPE tissue samples, may be over-fragmented or have modifications that adversely affect library preparation processes. Use the Agilent NGS FFPE QC Kit to determine the precise quantity of amplifiable DNA in the sample and allow direct normalization of input DNA amount.
- ✔ Performance of the solid-phase reversible immobilization (SPRI) purification step may be poor. Verify the expiration date for the vial of AMPure XP beads used for purification. Adhere to all bead storage and handling conditions recommended by the manufacturer. Ensure that the beads are kept at room temperature for at least 30 minutes before use. Use freshly-prepared 70% ethanol for each SPRI procedure.

If solids observed in the End Repair-A Tailing Buffer

- ✔ Vortex the solution at high speed until the solids are dissolved. The observation of solids when first thawed does not impact performance, but it is important to mix the buffer until all solutes are dissolved.

If pre-capture library fragment size is larger than expected in electropherograms

- ✔ Shearing may not be optimal. For intact, high-quality DNA samples, ensure that shearing is completed using the two-round shearing protocol provided, including all spinning and vortexing steps.
- ✔ Any bubbles present on the microTUBE filament may disrupt complete shearing. Spin the microTUBE for 30 seconds before the first round of shearing to ensure that any bubbles are released.

If pre-capture library fragment size is different than expected in electropherograms

- ✔ FFPE DNA pre-capture libraries may have a smaller fragment size distribution due to the presence of DNA fragments in the input DNA that are smaller than the target DNA shear size.
- ✔ DNA fragment size selection during SPRI purification depends upon using the correct ratio of sample to AMPure XP beads. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color. Verify that you are using the bead volume specified on **page 51** for library preparation purification and pre-capture purification. If the library preparation runset and pre-capture purification protocols are not run on the same day, be sure to seal the AMPure XP bead source plate and store at 4°C.

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 66** to **page 67**. The presence of excessive adaptor-dimers in the samples may be associated with reduced yield of pre-capture libraries.
- ✔ For whole-genome sequencing (not specifically supported by this protocol), samples with an adaptor-dimer peak must be subjected to an additional round of SPRI-purification. To complete, first dilute the sample to 50 µL with nuclease free water, then run the AMPureXP_XT_LI_ILM (Pre-Capture PCR) protocol.

If yield of post-capture libraries is low

- ✔ PCR cycle number may require optimization. Repeat library preparation and target enrichment for the sample, increasing the post-capture PCR cycle number by 1 to 2 cycles.
- ✔ The RNA Probe Capture Library 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 Capture Library Hybridization Mix is kept on ice until it is dispensed into the master mix source plate, as directed on **page 75**, 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. Before removing an aliquot of beads for the purification step, mix the beads until the suspension appears homogeneous and consistent in color and verify that you are using the bead volume specified for post-capture purification on **page 94**.

If low % on-target is observed in library sequencing results

- ✔ Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a thermal cycler in close proximity to the Bravo NGS Workstation to retain the 65°C sample temperature during transfer step (**step 28** on **page 81**).

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. 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 51** on page 73).

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In This Book

This guide contains information to run the SureSelect^{XT} Low Input protocol using automation protocols provided with the Agilent NGS Workstation Option B.

Version G3, December 2022

G9703-90010

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