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

Automated using Agilent NGS Bravo Option A

Protocol

Version C1, December 2022

SureSelect platform manufactured with Agilent SurePrint technology.

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

Notices

Manual Part Number

G9703-90020

Edition

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Acknowledgment

Technical Support

For US and Canada

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Or send an e-mail to:
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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 Bravo Option A.

1 Before You Begin

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

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

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

3 Sample Preparation

This chapter describes the steps to prepare 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 guidelines for sequencing sample preparation.

6 Appendix: Using FFPE-derived DNA Samples

This chapter described 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 C1

- Design ID information added to **Table 3** on page 12 for pre-designed SureSelect probes.
- Updates to downstream sequencing support information (see **page 89** to **page 96**). Key updates include guidelines for demultiplexing using Illumina's BCL Convert software (see **page 90**) and support for Agilent's new CReaK tool, replacing the LocatIt tool in AGeNT v3.0 (see **page 96**).
- 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 95**).
- New *Note* on cross- platform index equivalence on **page 41**.
- Update to **Notice to Purchaser**.

What's New in Version C0

- Support for SureSelect XT HS Human All Exon V8 Probe, SureSelect XT HS Human All Exon V8+UTR Probe, and SureSelect XT HS Human All Exon V8+NCV Probe (see **Table 3** on page 12).
- Updates to downstream sequencing platform and kit support information (**Table 69** on page 90).
- New recommendation regarding the use of compression pads with the thermal cycler (see **"Procedural Notes"** on page 10).
- Updates to thermal cycler recommendations (see **Table 4** on page 13) and usage instructions.
- Support for 5200 Fragment Analyzer and 4150 TapeStation instruments (see footnote to **Table 4** on page 13. Also see **page 53** and **page 86** for instructions.
- Updates to the recommendations regarding use of the molecular barcodes. See third paragraph on **page 29**.
- Updates to the Agilent NGS Bravo components user guide part numbers (see **Table 6** on page 18).
- Updated information on the pipette head options and fluid transfer capabilities of the Bravo platform (see **page 18**).
- Updated document look and feel.

What's New in Version B0

- Support for revised SureSelect custom probe products, produced using an updated manufacturing process beginning August, 2020 (see **Table 3** on page 12). Custom probes produced using the legacy manufacturing process are also fully supported by the protocols in this document. Probe nomenclature throughout document was updated.
- Support for separately-purchased SureSelect XT Low Input Reagent Kit G9703A or G9703B and Probe (see **Table 1** on page 11 and **Table 3** on page 12). Bundled SureSelect XT Low Input Reagent Kit + Probe products (Agilent p/n G9507A-S and G9508A-S) are also supported by the protocols in this document; see **page 104** for information on available Reagent Kit + Probe bundles.
- Updates to *Materials Required* including updated ordering information for Dynabeads MyOne Streptavidin T1 beads and AMPure XP Kits (**Table 1** on page 11) and for Qubit Fluorometer (**Table 4** on page 13).

- Updates to *Optional Materials* in **Table 5** on page 15, including removal of ethylene glycol supplier information (see **page 32** for related update to DNA shearing set up instructions).
- Updates to **step 3** on **page 30** and **step 3** on **page 31** to include formulation of Low TE Buffer.
- Corrections to one or more *Total Volume* values in **Table 27**, **Table 40**, **Table 44**, **Table 45**, and **Table 60**. Amounts of reagents combined to prepare the mixtures in the affected tables are unchanged.
- Updates to Technical Support contact information (see **page 2**).
- Updates to Notice to Purchaser (see **page 2**).

Content

1 Before You Begin

Make sure you have the most current protocol. Go to agilent.com and search for G9703-90020.

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 using the Agilent NGS Bravo Option A. For automated sample processing using the Agilent NGS Workstation Option B, see publication G9703-90010. 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 Bravo to allow rapid and efficient plate transfer.
- If your thermal cycler is compatible with the use of compression pads, add a compression pad whenever you load a plate that was sealed with the PlateLoc thermal microplate sealer. The pad improves contact between the plate and the heated lid of the thermal cycler.
- Use best-practices to prevent PCR product contamination of samples throughout the workflow:
  1. Assign separate pre-PCR and post-PCR pipettors, supplies, and reagents. In particular, never use materials designated to post-PCR segments for the pre-PCR segments of the workflow. For the pre-PCR workflow steps, 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.
- For each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of domed caps. Cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler and from other procedural steps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during thermal cycler incubation steps.
- In general, follow Biosafety Level 1 (BL1) 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.

Required Reagents

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.

Table 2 Required Reagents--FFPE DNA Samples Only

Description	Vendor and part number
FFPE DNA integrity assessment system: Agilent NGS FFPE QC Kit 16 reactions 96 reactions	 Agilent p/n G9700A p/n G9700B
OR	
TapeStation Genomic DNA Analysis Consumables: Genomic DNA ScreenTape Genomic DNA Reagents	Agilent p/n 5067-5365 p/n 5067-5366
QIAamp DNA FFPE Tissue Kit, 50 Samples	Qiagen p/n 56404
Deparaffinization Solution	Qiagen p/n 19093

Table 3 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 104** 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.

Required Equipment

Table 4 Required Equipment for Automated SureSelect^{XT} Low Input Target Enrichment

Description	Vendor and part number
Agilent NGS Bravo Option A Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5522A (VWorks software version 11.3.0.1195)
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
PlateLoc Thermal Microplate Sealer with Small Hotplate	Agilent p/n G5402#226
Clear Peelable Seal plate seals (for use with the PlateLoc Thermal Plate Sealer)	Agilent p/n 16985-001
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
Covaris Sample Preparation System	Covaris model E220
Covaris microTUBE sample holders	Covaris p/n 520045
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

Table 4 Required Equipment for Automated SureSelect^{XT} Low Input Target Enrichment (continued)

Description	Vendor and part number
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 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
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Plate centrifuge	Labnet International MPS1000 Mini Plate Spinner p/n C1000 or equivalent
Pipettes (multichannel pipette and P10, P20, P200 and P1000 pipettes)	Pipetman or equivalent
Vortex mixer	
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	

* 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 p/n 5067-5603, are required only when sample qualification is performed using VWorks automation protocols **05 TS_D1000** and **12 TS_HighSensitivity_D1000**.

Optional Reagents and Equipment

Table 5 Supplier Information for Optional Materials

Description	Vendor and part number
High-quality gDNA purification system, for example: QIAamp DNA Mini Kit 50 Samples 250 Samples	 Qiagen p/n 51304 p/n 51306
Tween 20	Sigma-Aldrich p/n P9416-50ML
Optical Caps, 8× strip (flat)	Agilent p/n 401425*

* Flat strip caps may be used instead of domed strip caps for protocol steps performed outside of the thermal cycler. Adhesive film may be applied over the flat strip caps for improved sealing properties.

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

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

About the NGS Bravo Option A

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

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

Table 6 Agilent NGS Bravo User Guide reference information

Device	User Guide part number
Bravo Platform	SD-V1000376 (previously G5562-90000)
VWorks Software (version 11.3.0.1195)	G5415-90063
PlateLoc Thermal Microplate Sealer	G5402-90001

About the Bravo Platform

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

Bravo Platform Deck

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

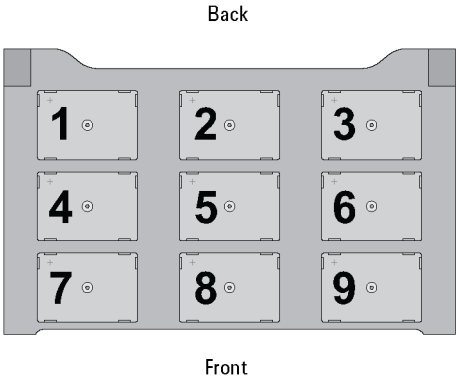


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

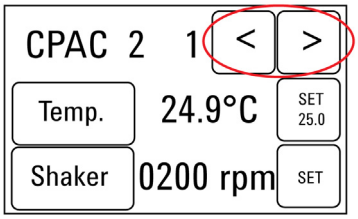
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

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

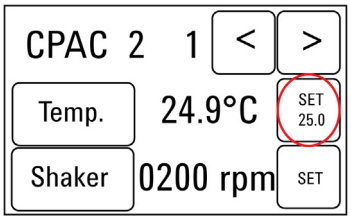
Table 7 Inheco Multi TEC Control touchscreen designations

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

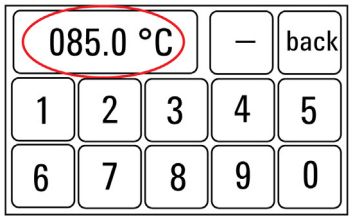
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



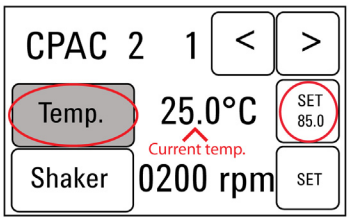
2 To set the temperature of the selected block, press the SET button.



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



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



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

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

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

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

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

3 Press the **START** button.

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

VWorks Automation Control Software

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

NOTE

The instructions in this manual are compatible with VWorks software version 11.3.0.1195.

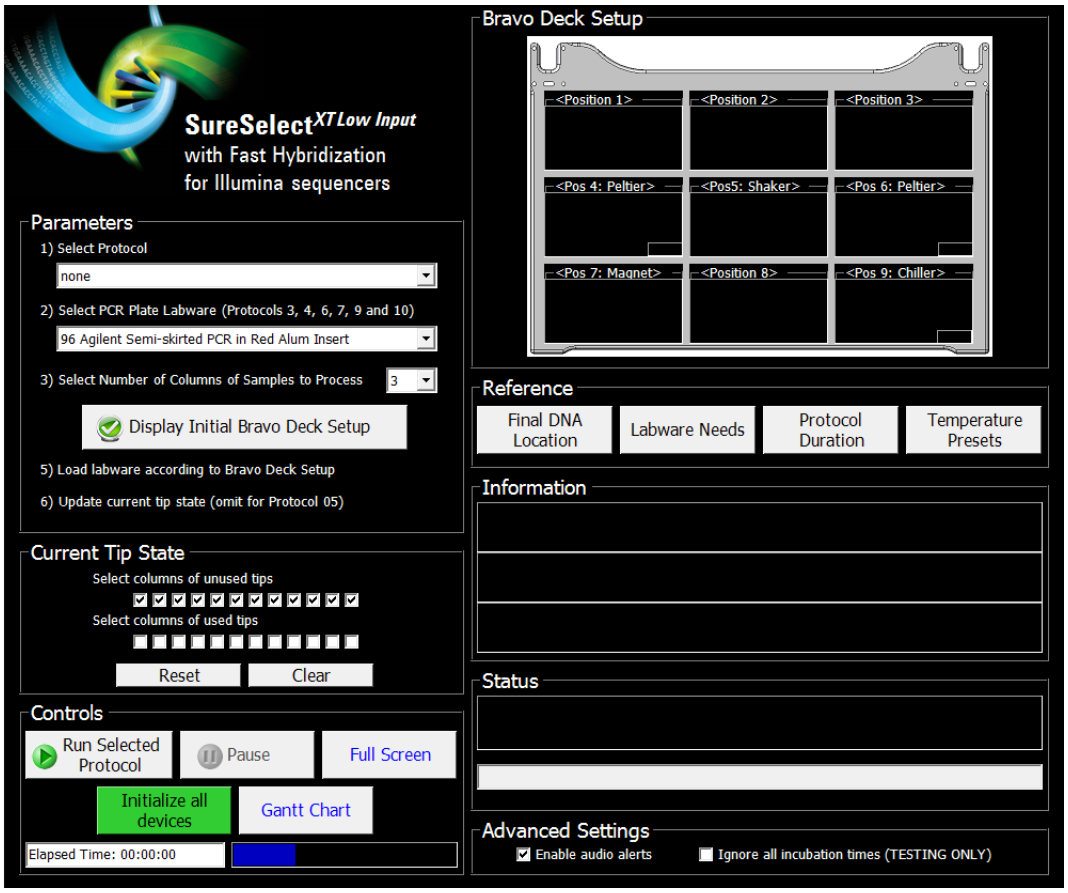
If you have questions about VWorks version compatibility, please contact service.automation@agilent.com.

Logging in to the VWorks software

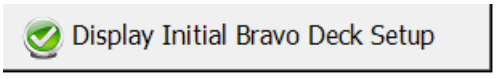
1. Double-click the VWorks icon or the XT_LI_ILM_v.A1.0.1.VWForm shortcut on the Windows desktop to start the VWorks software.
2. If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
3. In the User Authentication dialog, type your VWorks user name and password, and click **OK**. (If no user account is set up, contact the administrator.)

Using the XT_LI_ILM_v.A1.0.1.VWForm to setup and start a run

Use the VWorks form XT_LI_ILM_v.A1.0.1.VWForm, shown below, to set up and start each SureSelect automation protocol.



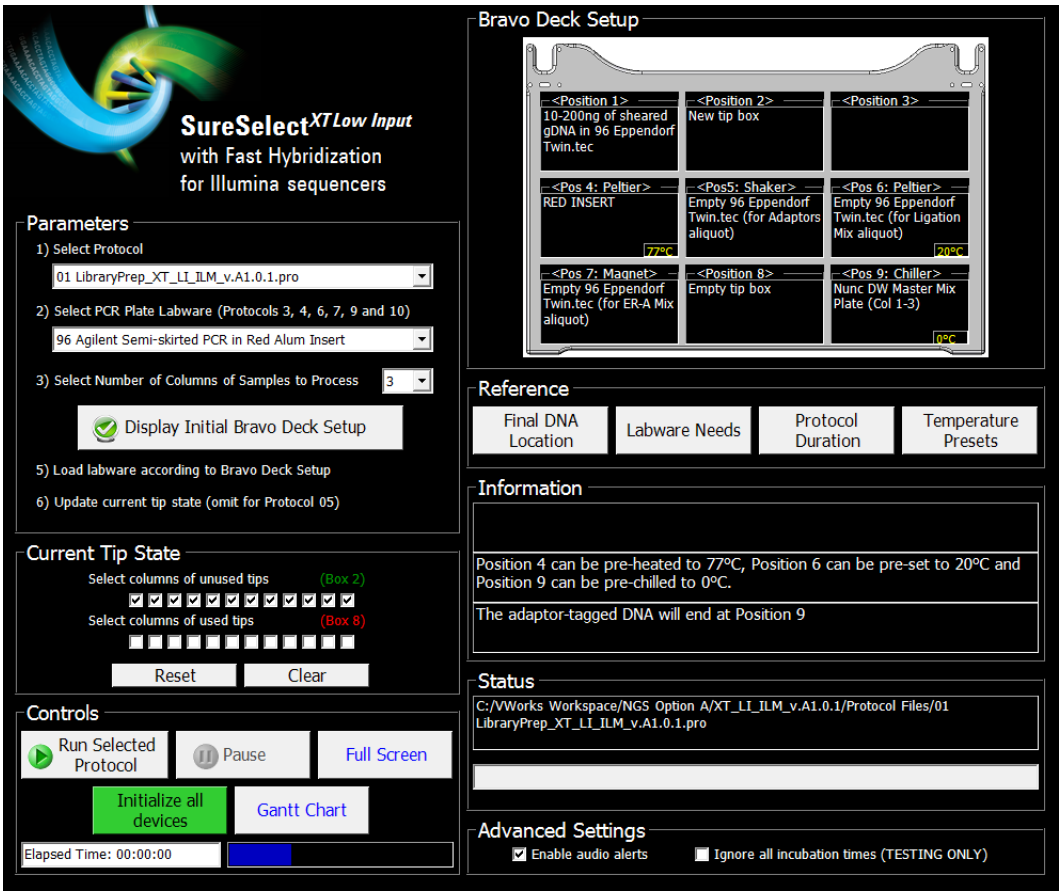
1. Open the form using the XT_LI_ILM_v.A1.0.1.VWForm shortcut on your desktop.
2. Use the drop-down menus on the form to select the appropriate SureSelect workflow step and other Parameters for the run.
3. Once all run parameters have been specified on the form, click **Display Initial Bravo Deck Setup**.



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

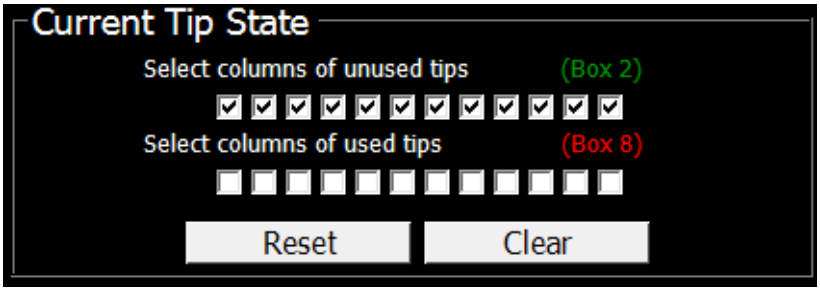
Load the Bravo Deck with labware and reagents as specified in the **Bravo Deck Setup** region of the form.

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



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

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



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

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

NOTE

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

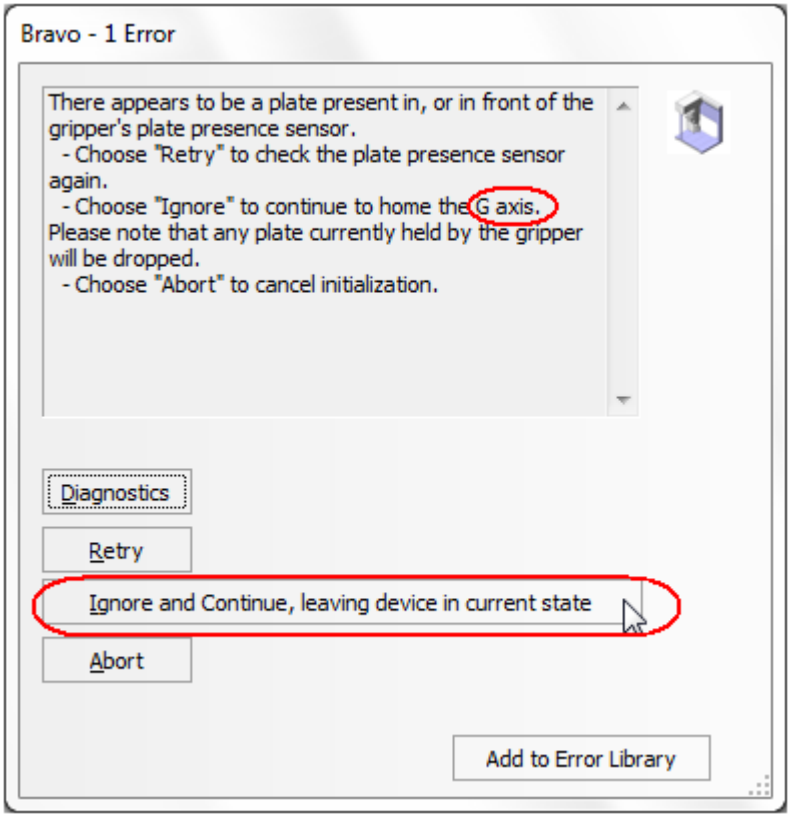
You can use partial tip boxes for NGS Bravo Option A automation protocols, as long as positions of available tips are accurately indicated during run setup.

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

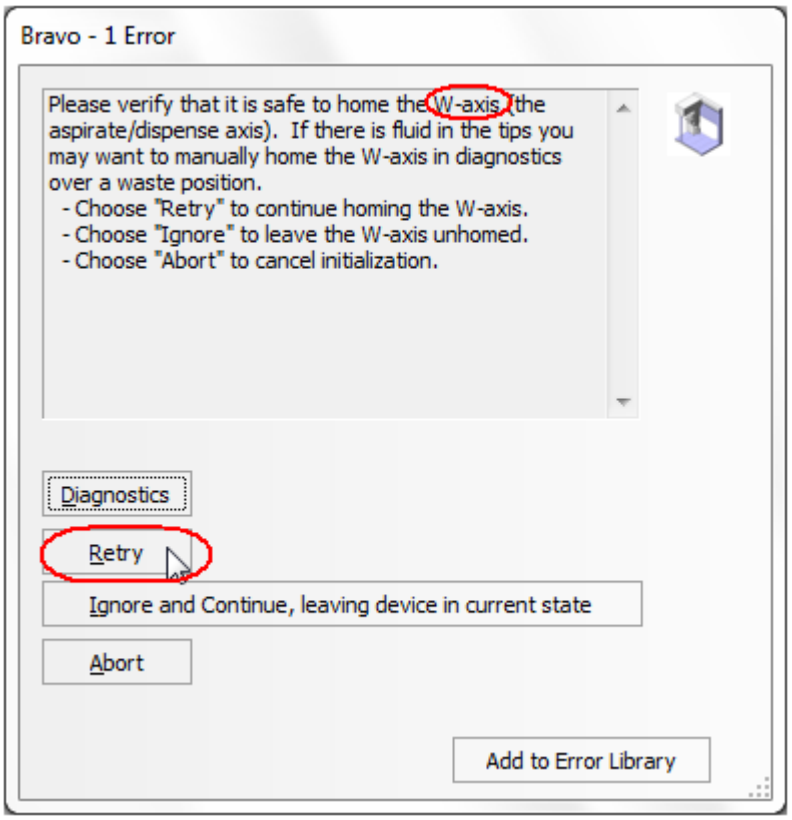
Error messages encountered at start of run

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

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



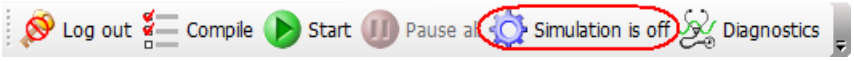
2 If you encounter the W-axis error message shown below, select **Retry**.



Verifying the Simulation setting

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

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



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

NOTE

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

Overview of the SureSelect Target Enrichment Procedure

Figure 2 summarizes the SureSelect^{XT} 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 8** 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.

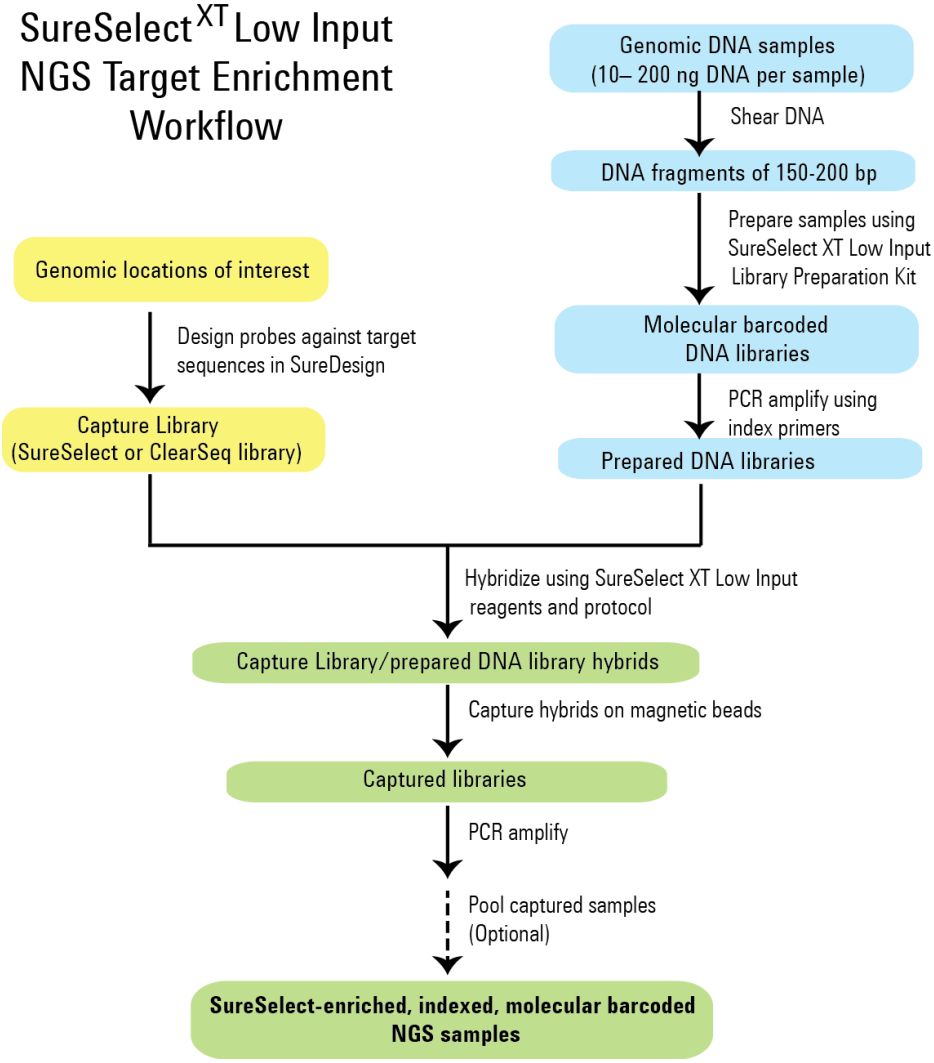


Figure 2 Overall sequencing sample preparation workflow.

Table 8 Overview of VWorks protocols

Workflow Step (Protocol Chapter)	Substep	VWorks Protocols Used for Agilent NGS Bravo automation
Sample Preparation	Prepare molecular-barcoded DNA libraries	01 LibraryPrep_XT_LI_ILM
	Purify DNA libraries using AMPure XP beads	02 Cleanup_LibPrep_XT_LI_ILM
	Amplify DNA libraries	03 Pre-CapPCR_XT_LI_ILM
	Purify amplified libraries using AMPure XP beads	04 Cleanup_Pre-CapPCR_XT_LI_ILM
	Analyze amplified libraries using Agilent TapeStation platform	05 TS_D1000
Hybridization	Aliquot 500-1000 ng of prepped libraries for hybridization	06 Aliquot_Libraries
	Hybridize prepped DNA to Capture Library (target enrichment)	07 Hyb_XT_LI_ILM
	Capture the DNA/Capture Library hybrids	08 SSELCapture_XT_LI_ILM
	Wash the captured DNA	09 SSELWash_XT_LI_ILM
Post-Hybridization	Amplify target-enriched DNA libraries	10 Post-CapPCR_XT_LI_ILM
	Purify enriched, amplified libraries using AMPure XP beads	11 Cleanup_Post-CapPCR_XT_LI_ILM
	Analyze final libraries using Agilent TapeStation platform	12 TS_HighSensitivity_D1000

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 9 Columns to Samples Equivalency

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

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

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

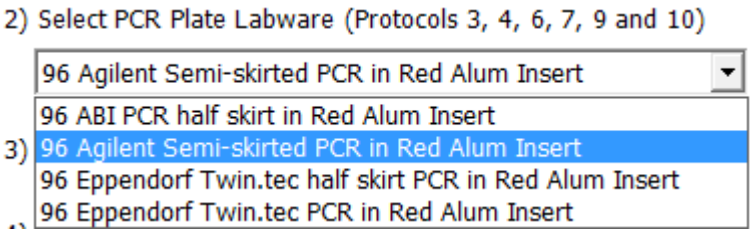
- The NGS Bravo processes samples column-wise beginning at column 1. gDNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- Samples are indexed during pre-capture amplification (see **Figure 2**). Assign each sample to the appropriate indexing primer during experimental design, and place the sample in the well corresponding to its assigned primer. See **Table 80** and **Table 81** on page 105 for indexing primer plate maps.
- At the hybridization step (see **Figure 2**), you can add a different SureSelect or ClearSeq Capture Library to each row of the plate. Plan your experiment such that each prepared DNA library plate position corresponds to the appropriate Capture Library row in the sample plate.
- For post-capture amplification (see **Figure 2**), different Capture Libraries can require different amplification cycle numbers, based on sizes of the captured targets. It is most efficient to process similar-sized Capture Libraries on the same plate. See **Table 59** on page 77 to determine which Capture Libraries 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 Bravo to allow rapid and efficient plate transfer.
- Several workflow steps require that the sample plate be sealed using the PlateLoc thermal microplate sealer and then centrifuged to collect any dispersed liquid. To maximize efficiency, locate the centrifuge in close proximity to the Agilent NGS Bravo.

PCR Plate Type Considerations

Automation protocols include several liquid-handling steps in which reagents are dispensed to PCR plates in preparation for transfer to a thermal cycler. For these steps you must specify the PCR plate type to be used on the XT_LI_ILM_v.A1.0.1.VWForm to allow correct configuration of the liquid handling components for the PCR plate type. 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 10**.



CAUTION

The plates listed in **Table 10** 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 10**, even if they are compatible with your chosen thermal cycler.

Table 10 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 25. This section contains instructions for automated gDNA library preparation for the Illumina paired-read sequencing platform using the Agilent NGS Bravo Option A. 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**, "Appendix: Using FFPE-derived DNA Samples" on **page 97**.

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

Step 1. Prepare and analyze quality of genomic DNA samples

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.

3 Prepare each DNA sample for the library preparation protocol by diluting 10 ng to 200 ng gDNA 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.

Additional qualification of DNA samples is not required for DNA derived from fresh biological samples. Proceed to **"Step 2. Shear the DNA"** on page 32.

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 11**.

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 11 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 12** to determine the recommended amount of input DNA for the sample.

Table 12 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.

3 Prepare each FFPE DNA sample for the library preparation protocol by diluting the appropriate amount of gDNA 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. See **Table 11** or **Table 12** above for FFPE DNA input guidelines based on the measured DNA quality in each sample.

Vortex each sample dilution to mix, then spin briefly to collect the liquid. Keep samples on ice.

Step 2. Shear the DNA

In this step, the 50-µL gDNA samples are 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 130-µl Covaris microTUBE 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 Complete the DNA shearing steps below for each of the gDNA samples.

   Each high-quality DNA sample or FFPE DNA sample should contain 10–200 ng gDNA (adjusted as required for DNA integrity) in 50 µL of 1X Low TE Buffer.

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

Transfer the 50-µL DNA sample into a Covaris microTUBE, using a tapered pipette tip to slowly transfer the sample through the pre-split septum of the cap.

- **a** Spin the microTUBE for 30 seconds to collect the liquid and to remove any bubbles from the bottom of the tube.
- **b** Secure the microTUBE in the tube holder and shear the DNA with the settings in **Table 13**.

Table 13 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 microTUBE for 10 seconds
- Vortex the microTUBE at high speed for 5 seconds
- Spin the microTUBE for 10 seconds
- Shear for additional 120 seconds
- Spin the microTUBE for 10 seconds
- Vortex the microTUBE at high speed for 5 seconds
- Spin the microTUBE for 10 seconds

c After completing the shearing step(s), put the Covaris microTUBE back into the loading and unloading station.

d While keeping the snap-cap on, insert a pipette tip through the pre-split septum, then slowly remove the sheared DNA.

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

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

NOTE

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

Step 3. Prepare adaptor-ligated libraries

In this step, automation protocol **01 LibraryPrep_XT_LI_ILM** is used to complete the DNA end modification steps required for SureSelect target enrichment, including end-repair, dA-tailing, and ligation of the molecular-barcoded adaptor.

This step uses the components listed in **Table 14**. Thaw and mix each component as directed in **Table 14** 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.

For use in the next automation protocol on **page 46**, remove the AMPure XP beads from cold storage and equilibrate to room temperature.

Table 14 Reagents to be thawed or equilibrated to room temperature 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 35
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 35
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 35
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 35
Adaptor Oligo Mix (white cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Thaw on ice then keep on ice	Vortexing	page 36

Prepare the NGS Bravo

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

2 Pre-set the temperature of Bravo deck positions 4, 6, and 9 as indicated in **Table 15**. See **page 19** to **page 20** for more information on how to do this step.

Table 15 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
4	79°C	Inheco Multi TEC control touchscreen (CPAC 2-1)
6	20°C	Inheco Multi TEC control touchscreen (CPAC 2-2)
9	0°C	ThermoCube control panel

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

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

4 Prepare the appropriate volume of End Repair/dA-Tailing master mix, using volumes listed in **Table 16** 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 16 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	1768 µL
End Repair-A Tailing Enzyme Mix (orange cap)	4 µL	51 µL	85 µL	119 µL	153 µL	221 µL	442 µL
Total Volume	**20 µL**	**255 µL**	**425 µL**	**595 µL**	**765 µL**	**1105 µL**	**2210 µL**

Prepare the Ligation master mix

5 Prepare the appropriate volume of Ligation master mix, using volumes listed in **Table 17** 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 17 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	2541.5 µL
T4 DNA Ligase (blue cap)	2 µL	25.5 µL	42.5 µL	59.5 µL	76.5 µL	110.5 µL	221.0 µL
Total Volume	**25 µL**	**318.8 µL**	**531.3 µL**	**743.8 µL**	**956.3 µL**	**1381.3 µL**	**2762.5 µL**

Prepare the Adaptor Oligo Mix dilution

6 Prepare the appropriate volume of Adaptor Oligo Mix dilution, according to **Table 18**. Mix well using a vortex mixer and keep on ice.

Table 18 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	533.0 µL
Total Volume	**7.5 µL**	**127.5 µL**	**191.3 µL**	**255.0 µL**	**318.8 µL**	**430.5 µL**	**799.5 µL**

Prepare the master mix source plate

7 In a Nunc DeepWell plate, prepare the master mix source plate containing the mixtures prepared in **step 4** through **step 6**. Add the volumes indicated in **Table 19** 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 3**.

Table 19 Preparation of the Master Mix Source Plate for 01 LibraryPrep_XT_LI_ILM

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 1 (A1-H1)	31.0 µL	52.0 µL	73.0 µL	94.0 µL	136.5 µL	273.0 µL
Ligation master mix	Column 2 (A2-H2)	36.0 µL	62.0 µL	88.0 µL	114.0 µL	169.0 µL	338.0 µL
Adaptor Oligo Mix dilution	Column 3 (A3-H3)	15.0 µL	22.5 µL	30.0 µL	37.5 µL	52.5 µL	97.5 µL

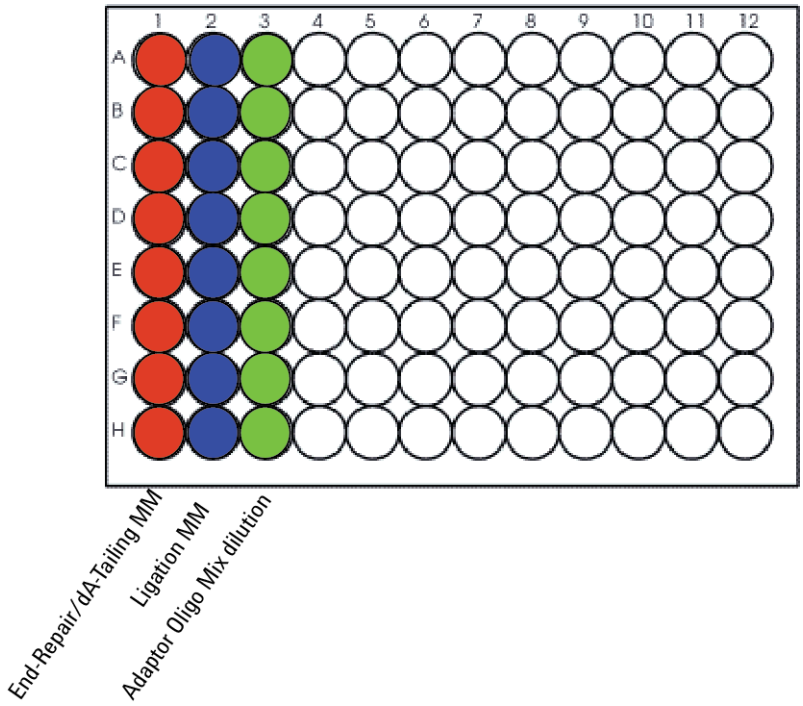


Figure 3 Configuration of the master mix source plate for **01 LibraryPrep_XT_LI_ILM**

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. Keep the master mix source plate on ice.

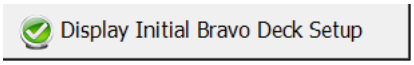
NOTE The presence of bubbles in source plate solutions may cause inaccurate volume transfer by the Bravo liquid handling platform. Ensure that the source plate is sealed and centrifuged prior to use in a run.

Setup and run VWorks protocol 01 LibraryPrep_XT_LI_ILM

10 On the SureSelect setup form, under **Select Protocol to Run**, select **01 LibraryPrep_XT_LI_ILM**.

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

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



13 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 20.

Table 20 Initial Bravo deck configuration for 01 LibraryPrep_XT_LI_ILM

Location	Content
1	Eppendorf plate containing sheared gDNA samples
2	New tip box
3	–(empty)–
4	Empty red insert
5	Empty Eppendorf twin.tec plate
6	Empty Eppendorf twin.tec plate
7	Empty Eppendorf twin.tec plate
8	Empty tip box
9	Library Prep Master Mix Source Plate, unsealed

14 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

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

Running the 01 LibraryPrep_XT_LI_ILM protocol takes approximately 90 minutes. Once complete, the adaptor-tagged DNA samples are located in the Eppendorf plate at position 9 of the Bravo deck.

Step 4. Purify adaptor-ligated DNA using AMPure XP beads

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

In this step the NGS Bravo combines the adaptor-ligated samples with AMPure XP beads and then collects and washes the bead-bound DNA.

Prepare the NGS Bravo and reagents

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

2 Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in **Table 21**. See **page 19** for more information on how to do this step.

Table 21 Bravo Deck Temperature Presets

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

3 Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.

4 Prepare a Nunc DeepWell source plate for the beads by adding 80 µL of homogeneous AMPure XP beads per well, for each well to be processed.

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

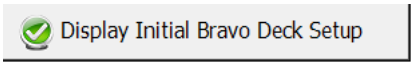
6 Prepare a separate Thermo Scientific Matrix reservoir containing 45 mL of freshly-prepared 70% ethanol.

Setup and Run VWorks protocol 02 Cleanup_LibPrep_XT_LI_ILM

7 On the SureSelect setup form, under **Select Protocol**, select **02 Cleanup_LibPrep_XT_LI_ILM**.

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

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



10 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 22.

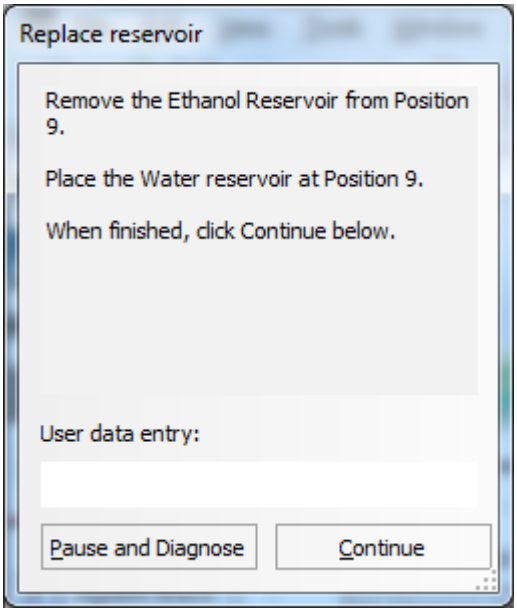
Table 22 Initial Bravo deck configuration for 02 Cleanup_LibPrep_XT_LI_ILM

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
2	New tip box
3	–(empty)–
4	–(empty)–
5	AMPure XP beads in Nunc DeepWell plate (80 µL beads per processing well)
6	Adaptor-tagged DNA samples in Eppendorf twin.tec plate
7	–(empty)–
8	Empty tip box
9	70% ethanol in Matrix reservoir

11 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

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

Running the 02 Cleanup_LibPrep_XT_LI_ILM protocol takes approximately 45 minutes. An operator must be present during the run to complete tip box replacement and to transfer the water reservoir to the Bravo deck, when directed by the VWorks prompt shown below.



Once the protocol is complete, the purified DNA samples are located in the Nunc DeepWell plate at position 7 of the Bravo deck.

Step 5. Amplify adaptor-ligated libraries

In this step, the Agilent NGS Bravo completes the liquid handling steps for amplification of the adaptor-ligated DNA samples using automation protocol **03 Pre-CapPCR_XT_LI_ILM**. Afterward, you transfer the PCR plate to a thermal cycler for amplification.

This step uses the components listed in **Table 23**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 23 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 43**
5× Herculase II Reaction Buffer (clear cap)	SureSelecty XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	**page 43**
100 mM dNTP Mix (green cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	**page 43**
Forward Primer (brown cap)	SureSelect XT HS and XT Low Input Library Preparation Kit for ILM (Pre PCR), –20°C	Vortexing	**page 43**
SureSelect XT Low Input Index Primers	SureSelect XT Low Input Index Primers for ILM (Pre PCR),* –20°C	Vortexing	**page 43**

* 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*).

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 NGS Bravo

1 Retain the purified DNA samples in the Nunc DeepWell plate at position 7 of the Bravo deck. Clear the remaining Bravo deck of all plates and tip boxes.

2 Pre-set the temperature of Bravo deck positions 6 and 9 as indicated in **Table 24**. See **page 19** to **page 20** for more information on how to do this step.

Table 24 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
6	4°C	Inheco Multi TEC control touchscreen (CPAC 2-2)
9	0°C	ThermoCube control panel

3 Place a red PCR plate insert at Bravo deck position 6 and a silver deep well plate insert at Bravo deck position 9.

Pre-program the thermal cycler

4 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 25**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the automation run.

Table 25 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 26**)	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 26 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

5 Prepare the appropriate volume of pre-capture PCR Master Mix, according to **Table 27**. Mix well using a vortex mixer and keep on ice.

Table 27 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	6.5 µL	82.9 µL	138.1 µL	193.4 µL	248.6 µL	359.1 µL	718.3µL
5× Herculase II Reaction Buffer (clear cap)	10 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1105 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	55.3 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	110.5 µL
Total Volume	**20 µL**	**255.1 µL**	**425.0 µL**	**595.1 µL**	**765 µL**	**1105 µL**	**2210.1 µL**

6 Using the same Nunc DeepWell master mix source plate that was used for the 01 LibraryPrep_XT_LI_ILM run, add the volume of PCR Master Mix indicated in **Table 28** to all wells of column 4 of the master mix source plate. The final configuration of the master mix source plate is shown in **Figure 4**.

Table 28 Preparation of the Master Mix Source Plate for 03 Pre-CapPCR_XT_LI_ILM

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 4 (A4-H4)	31.0 µL	52.0 µL	73.0 µL	94.0 µL	136.5 µL	273.0 µL

NOTE If you are using a new DeepWell plate for the pre-capture PCR source plate, leave columns 1 to 3 empty and add the PCR Master Mix to column 4 of the new plate.

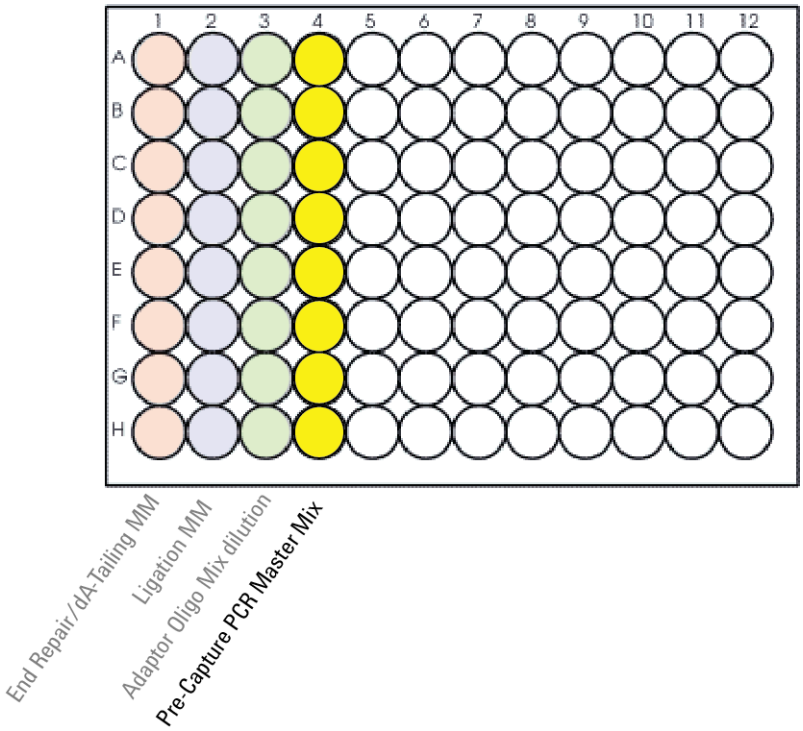


Figure 4 Configuration of the master mix source plate for **03 Pre-CapPCR_XT_LI_ILM**. Columns 1-3 were used to dispense master mix during the previous protocol.

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

8 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

9 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, combine 2 µL of the specific indexing primer assigned to the sample well with 3 µL of water. Keep the plate on ice.

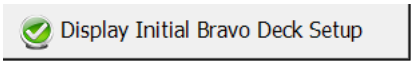
Setup and run VWorks protocol 03 Pre-CapPCR_XT_LI_ILM

10 On the SureSelect setup form, under **Select Protocol to Run**, select **03 Pre-CapPCR_XT_LI_ILM**.

11 Under **Select PCR plate Labware**, select the specific type of PCR plate to be used for thermal cycling (containing the index primers, as described in **step 9** above).

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 Bravo Deck Setup**.



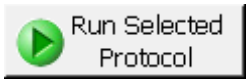
14 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in **Table 29**.

Table 29 Initial Bravo deck configuration for 03 Pre-CapPCR_XT_LI_ILM

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
2	New tip box
3	–(empty)–
4	–(empty)–
5	–(empty)–
6	Aliquotted indexes in PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
7	Purified adaptor-tagged DNA samples in Nunc DeepWell plate
8	Empty tip box
9	Master mix plate containing PCR Master Mix in Column 4 (unsealed), seated in silver insert

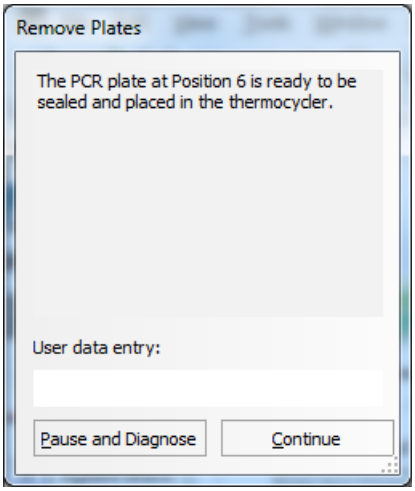
15 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

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



17 Running the 03 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.

18 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.



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

20 Before adding the samples to the preprogrammed thermal cycler, bring the temperature of the thermal block to 98°C by pressing the *Play* button to resume the thermal cycling program in **Table 25**. 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 6. Purify amplified DNA using AMPure XP beads

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

This step uses protocol **04 Cleanup_Pre-CapPCR_XT_LI_ILM**.

Prepare the NGS Bravo and reagents

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

2 Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in **Table 30**. See **page 19** for more information on how to do this step.

Table 30 Bravo Deck Temperature Presets

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

3 Verify that the AMPure XP bead suspension is at room temperature. (If necessary, allow the bead solution to come to room temperature for at least 30 minutes.) *Do not freeze the beads at any time*.

4 Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.

5 Prepare a Nunc DeepWell source plate for the beads by adding 50 µL of homogeneous AMPure XP beads per well, for each well to be processed.

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

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

8 Centrifuge the amplified DNA sample plate for 30 seconds to drive the well contents off the walls and plate seal.

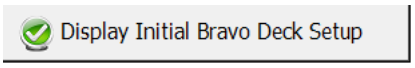
Setup and run VWorks 04 Cleanup_Pre-CapPCR_XT_LI_ILM

9 On the SureSelect setup form, under **Select Protocol**, select **04 Cleanup_Pre-CapPCR_XT_LI_ILM**.

10 Under **Select PCR plate Labware**, select the specific type of PCR plate used for pre-capture amplification.

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

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



13 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 31.

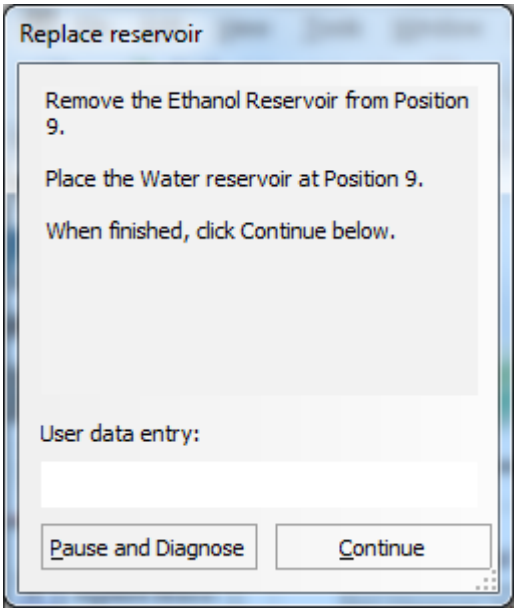
Table 31 Initial Bravo deck configuration for 04 Cleanup_Pre-CapPCR_XT_LI_ILM

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
2	New tip box
3	Empty Eppendorf plate
4	–(empty)–
5	AMPure XP beads in Nunc DeepWell plate (50 µL beads per processing well)
6	Amplified DNA libraries in unsealed PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
7	–(empty)–
8	Empty tip box
9	70% ethanol in Matrix reservoir

14 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

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

Running the 04 Cleanup_Pre-CapPCR_XT_LI_ILM protocol takes approximately 45 minutes. An operator must be present during the run to complete tip box replacement and to transfer the water reservoir to the Bravo deck, when directed by the VWorks prompt shown below.



Once the protocol is complete, the purified DNA samples are located in the Eppendorf plate at position 3 of the Bravo deck.

Step 7. 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 **05 TS_D1000**) and perform analysis on Agilent 4200 TapeStation. See **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and D1000 ScreenTape"** on page 49.
- 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 53.

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

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

This section describes use of automation protocol **05 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.

NOTE

If desired, the assay sample plate may also be prepared using manual liquid-handling steps as described in the D1000 Assay Quick Guide above, using 1 µl of each DNA sample and 3 µl of D1000 sample buffer, as indicated in the Guide. Follow the electropherogram analysis guidelines on **page 51**.

Prepare the NGS Bravo and Sample Buffer source plate

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
3. Turn off the ThermoCube device (see **page 20**) to restore position 9 of the Bravo deck to room temperature.
4. 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 32** to each well of column 1 of the plate.

Table 32 Preparation of the Sample Buffer Source Plate for 05 TS_D1000

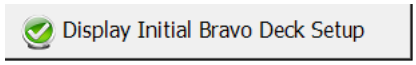
Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Eppendorf twin.tec Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
D1000 Sample Buffer	Column 1 (A1-H1)	11.0 µL	17.0 µL	23.0 µL	29.0 µL	41.0 µL	77.0 µL

Setup and Run VWorks protocol 05 TS_D1000

5 On the SureSelect setup form, under **Select Protocol to Run**, select **05 TS_D1000**.

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

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



8 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 33.

Table 33 Initial Bravo deck configuration for 05 TS_D1000

Location	Content
1	–(empty)–
2	New tip box
3	–(empty)–
4	Amplified pre-capture libraries in Eppendorf twin.tec plate (unsealed)
5	–(empty)–
6	Empty Eppendorf twin.tec plate
7	–(empty)–
8	Empty tip box
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 Bravo, 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.

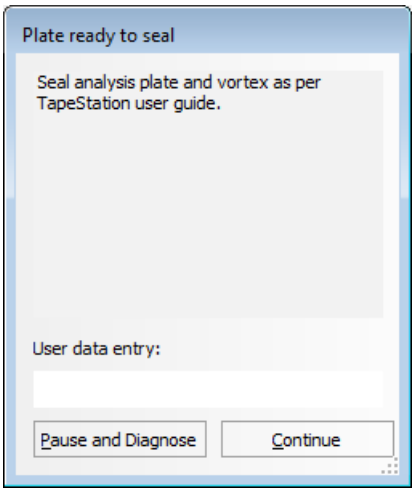
9 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

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

Running the 05 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 58**.

11 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

12 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.

13 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 5** (library prepared from high-quality DNA), **Figure 6** (library prepared from medium-quality FFPE DNA), and **Figure 7** (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 109** for additional considerations.

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

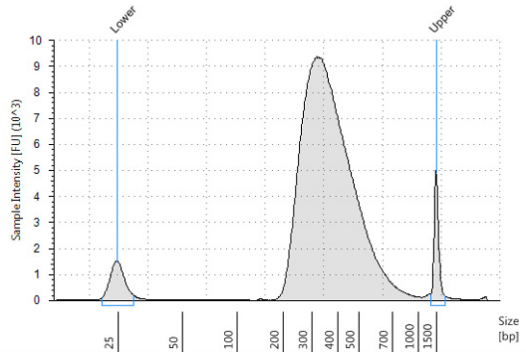


Figure 5 Pre-capture library prepared from a high-quality gDNA sample analyzed using a D1000 ScreenTape assay.

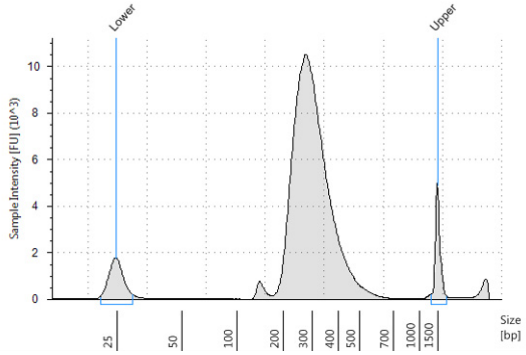


Figure 6 Pre-capture library prepared from a typical FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

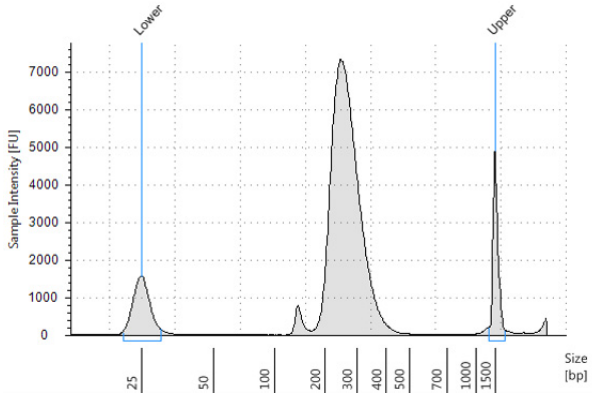


Figure 7 Pre-capture library prepared from a low-quality FFPE gDNA sample analyzed using a D1000 ScreenTape assay.

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 5** through **Figure 7**). **Table 34** includes links to assay instructions.

Table 34 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 Probe 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 49** to **page 53**, 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 06 Aliquot_Libraries is used to prepare a new sample plate containing the appropriate amount of each DNA sample for hybridization. Before running the automation protocol, you must create a table containing instructions for the NGS Bravo indicating the volume of each sample to aliquot, as described in the steps below.

1. Create a .csv (comma separated value) file with the headers shown in **Figure 8**. 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. For all empty wells on the plate, enter the value 0, as shown in **Figure 8**; 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 8 Sample spreadsheet for 1-column run.

NOTE

You can find a sample spreadsheet in the directory **C: > VWorks Workspace > NGS Option A> XT_LI_ILM_v.A1.0.1> Aliquot Library Input Files > Aliquot_Libraries_full_plate_template.csv.**

The Aliquot_Libraries_full_plate_template.csv file may be copied and used as a template for creating the .csv files for each Aliquot_Libraries 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 A/XT_LI_ILM_v.A1.0.1/Aliquot Library Input Files**.

Setup and Run VWorks protocol 06 Aliquot_Libraries

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

2 Pre-set the temperature of Bravo deck position 9 as indicated in **Table 35**. See **page 20** for more information on how to do this step.

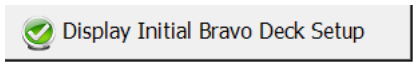
Table 35 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
9	0°C	ThermoCube control panel

3 On the SureSelect setup form, under **Select Protocol**, select **06 Aliquot_Libraries**.

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

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



6 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 36.

Table 36 Initial Bravo deck configuration for 06 Aliquot_Libraries

Location	Content
1	–(empty)–
2	–(empty)–
3	–(empty)–
4	–(empty)–
5	Empty Eppendorf plate
6	Empty tip box
7	–(empty)–
8	New tip box
9	Prepped library DNA in Eppendorf plate

CAUTION This protocol does not use the **Current Tip State** indicator function. Be sure to place a completely full box of tips at position 8 and a completely empty tip box at position 6.

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

8 When prompted by the dialog below, 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.

9 Remove the sample plate from the Bravo deck and use a vacuum concentrator to dry the samples at ≤ 45°C.

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

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

12 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 **07 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 37**. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin tubes briefly to collect the liquid.

Table 37 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 60**
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 61**
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 61**
Probe Capture Library	–80°C	Thaw on ice	**page 61**

Pre-program the thermal cycler

1 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 38**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the 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 38 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 Bravo steps†	1	65°C	Hold
4	Hybridization	60	65°C‡	1 minute
			37°C	3 seconds
5	Hold until start of Capture**	1	65°C†	Hold

* When setting up the thermal 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 Bravo deck during this Hold step when prompted by the VWorks software.

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

** Samples are held at 65°C until they are processed in the Capture automation protocol that begins on **page 68**.

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

Prepare the NGS Bravo

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

2 Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in **Table 39**. See **page 19** for more information on how to do this step.

Table 39 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
4	23°C	Inheco Multi TEC control touchscreen (CPAC 2-1)
6	23°C	Inheco Multi TEC control touchscreen (CPAC 2-2)

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

Prepare the Block master mix

4 Prepare the appropriate volume of Block master mix, on ice, as indicated in **Table 40**.

Table 40 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

5 Prepare the appropriate volume of Hybridization Buffer master mix, at room temperature, as indicated in **Table 41**.

Table 41 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

6 Prepare the appropriate volume of Capture Library Master Mix for each of the Probes that will be used for hybridization as indicated in **Table 42** to **Table 45**. 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 42** or **Table 43**) on **page 61**.

For runs that use different Probes for individual rows, prepare each master mix as described in Step b (**Table 44** or **Table 45**) on **page 62**.

a For runs that use a single Probe for all rows, prepare a Master Mix as described in **Table 42** or **Table 43**, according to the probe design size.

Table 42 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 43 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 44** or **Table 45**, according to the probe design size. The volumes listed in **Table 44** and **Table 45** 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 44 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 45 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

7 Using an Eppendorf twin.tec plate, prepare the hybridization master mix source plate at room temperature, containing the master mixes prepared in **step 4** to **step 6**. Add the volumes indicated in **Table 46** 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 9**.

Table 46 Preparation of the Master Mix Source Plate for 07 Hyb_XT_LI_ILM

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

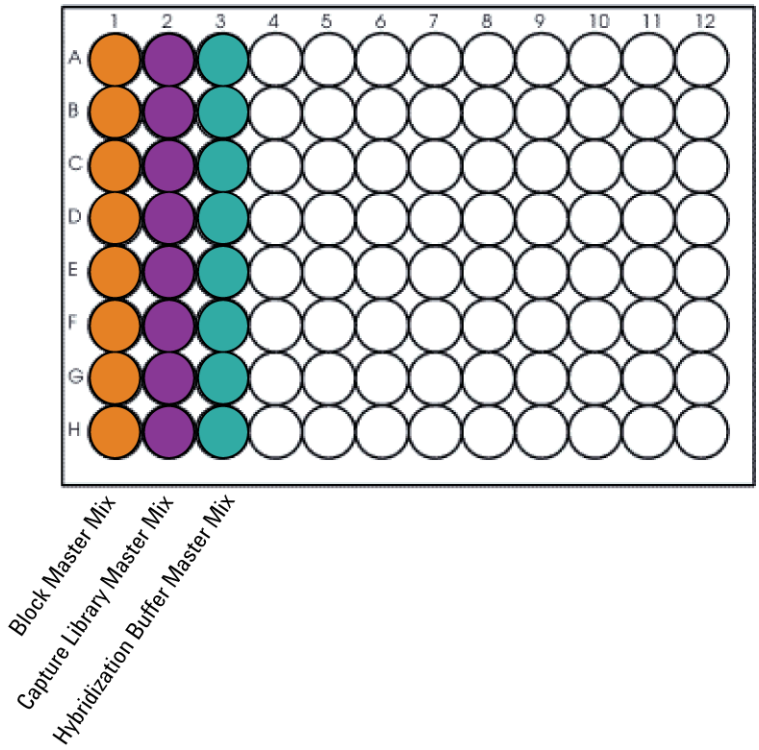


Figure 9 Configuration of the master mix source plate for **07 Hyb_XT_LI_ILM**.

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. Keep the master mix plate at room temperature.

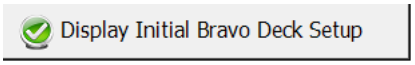
Setup and run VWorks protocol 07 Hyb_XT_LI_ILM

10 On the SureSelect setup form, under **Select Protocol,** select **07 Hyb_XT_LI_ILM**.

11 Under **Select PCR Plate Labware**, select the plate type to be used for the hybridization step (to be loaded at Bravo deck position 4).

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 Bravo Deck Setup**.



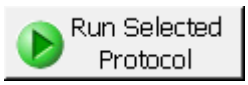
14 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 47.

Table 47 Initial Bravo deck configuration for 07 Hyb_XT_LI_ILM

Location	Content
1	–(empty)–
2	New tip box
3	–(empty)–
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
7	–(empty)–
8	Empty tip box
9	Prepped library aliquots in Eppendorf twin.tec plate (unsealed)

15 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

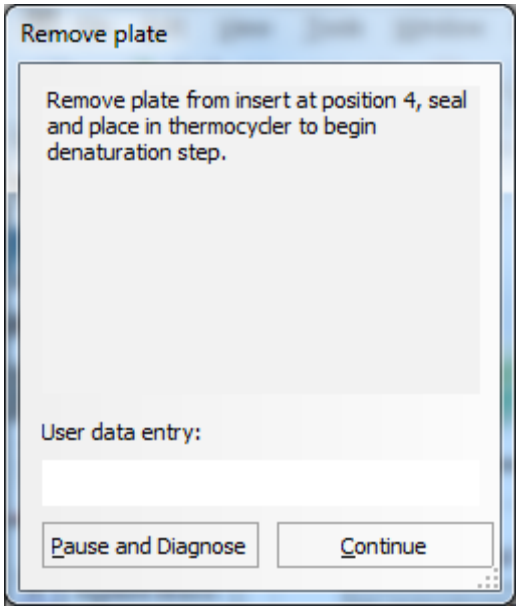
16 When setup and verification is complete, click **Run Selected Protocol**.



Running the 07 Hyb_XT_LI_ILM protocol takes approximately 30 minutes. An operator must be present during the run to complete tip box replacement and other labware transfer steps, as directed by the VWorks prompts detailed below.

The NGS Bravo 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.

17 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**.



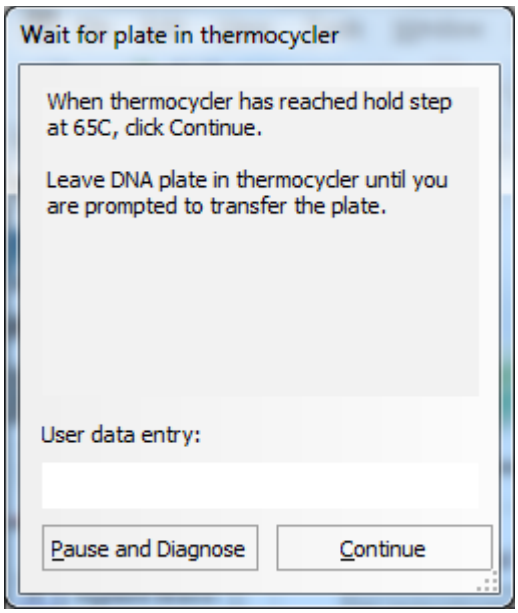
18 Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 sec.

19 Transfer the sealed plate to a thermal cycler and initiate the preprogrammed thermal cycling program described in **Table 38** on page 59.

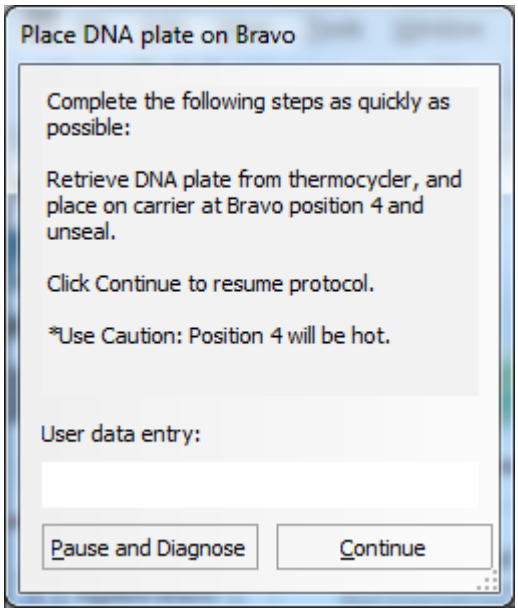
While the sample plate incubates on the thermal cycler, the NGS Bravo combines aliquots of the Capture Library Master Mix and Hybridization Buffer Master Mix.

CAUTION You must complete **step 20** to **step 24** quickly, and immediately after being prompted by the VWorks software. It is important that sample temperature remains approximately 65°C during transfers between the NGS Bravo and thermal cycler.

20 When the NGS Bravo 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 (Segment 3), click **Continue**. Leave the sample plate in the thermal cycler until you are notified to move it.



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

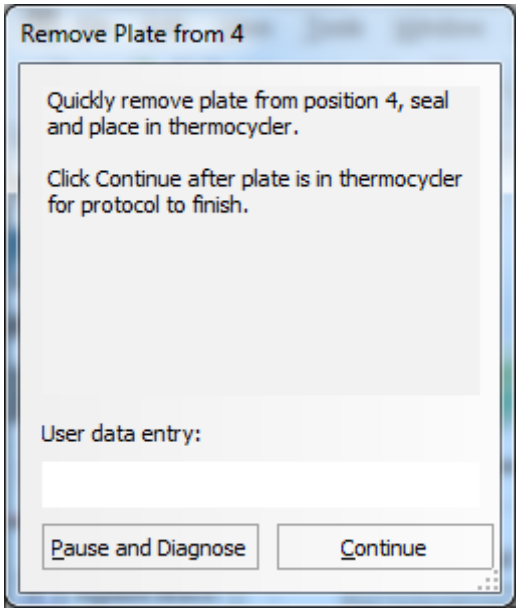


WARNING **Bravo deck position 4 will be hot.**

Use caution when handling components that contact heated deck positions.

The NGS Bravo transfers the Capture Library-Hybridization Buffer mixture to the wells of the PCR plate that contain the mixture of prepped gDNA samples and blocking agents.

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



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

24 Quickly transfer the plate back to the thermal cycler, held at 65°C. Press the *Play* button to initiate the hybridization segment of the pre-programmed thermal cycling program (segment 4 from **Table 38** on page 59). 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.

25 After initiating hybridization on the thermal cycler, click **Continue** on the VWorks screen to finish the NGS Bravo protocol.

During the thermal cycler incubation for hybridization (approximately 1.5-hour duration), complete the reagent and NGS Bravo setup steps for the capture automation protocol as described on **page 68** to **page 69**.

Step 3. Capture the hybridized DNA

This step uses automation protocol **08 SSELCapture_XT_LI_ILM** to automate capture of the gDNA-probe hybrids using streptavidin-coated magnetic beads. Setup tasks for the Capture protocol (**step 1**, below, through **step 16** on **page 70**) should be completed during the thermal cycler incubation for hybridization started on **page 67**.

This step uses the components listed in **Table 48**.

Table 48 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 68**
SureSelect Wash Buffer 2	SureSelect Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), RT	**page 69**
Dynabeads MyOne Streptavidin T1	Follow storage recommendations provided by supplier (see **Table 1** on page 11)	**page 68**

Prepare the NGS Bravo

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

2 Pre-set the temperature of Bravo deck position 4 as indicated in **Table 49**. See **page 19** for more information on how to do this step.

Table 49 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
4	66°C	Inheco Multi TEC control touchscreen (CPAC 2-1)

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

4 Place the silver Nunc DeepWell plate insert at Bravo deck position 6. This insert is required to facilitate heat transfer to DeepWell source plate wells.

Prepare the Dynabeads streptavidin beads and Wash Buffer 2 source plates

5 Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. The beads settle during storage.

6 Wash the magnetic beads.

a In a conical vial, combine the components listed in **Table 50**. The volumes below include the required overage.

Table 50 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

Table 50 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
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 device, such as the Dynal magnetic separator.

d Remove and discard the supernatant.

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

7 Resuspend the beads in SureSelect Binding buffer, according to **Table 51** below.

Table 51 Preparation of magnetic beads for 08 SSELCapture_XT_LI_ILM

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

8 Prepare 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.

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

10 Prepare a Nunc DeepWell source plate labeled *Wash #2*. For each well to be processed, add 1150 µL of SureSelect Wash Buffer 2. Place the Wash #2 source plate at position 6 of the Bravo deck, seated in the silver insert.

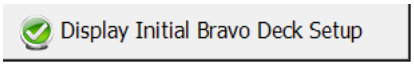
Setup VWorks protocol 08 SSELCapture_XT_LI_ILM

11 On the SureSelect setup form, under **Select Protocol**, select **08 SSELCapture_XT_LI_ILM**.

12 Under **Select PCR Plate Labware**, 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.

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

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



15 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 52.

Table 52 Initial Bravo deck configuration for 08 SSELCapture_XT_LI_ILM

Location	Content
1	–(empty)–
2	New tip box
3	–(empty)–
4	**Empty** red insert
5	Prepared Dynabeads streptavidin bead DeepWell source plate
6	*Wash #2* DeepWell source plate seated on silver Nunc DeepWell insert
7	–(empty)–
8	Empty tip box
9	–(empty)–

16 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

Run VWorks protocol 08 SSELCapture_XT_LI_ILM

Start the **08 SSELCapture_XT_LI_ILM** protocol upon completion of the hybridization incubation that was started on **page 67**, when the thermal cycler program reaches the 65°C Hold step in Segment 5.

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

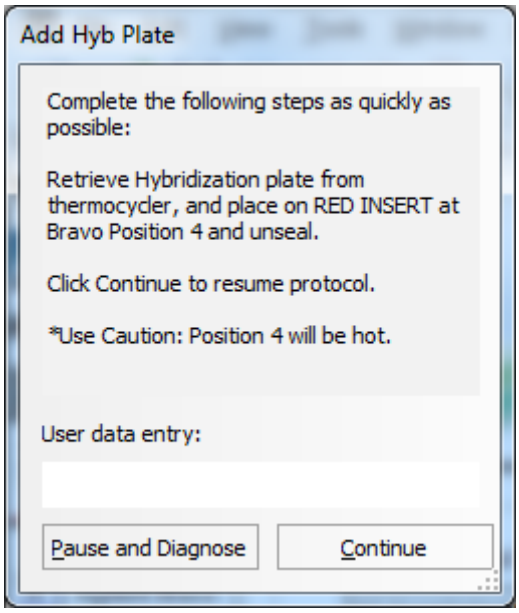
The total duration of the 08 SSELCapture_XT_LI_ILM protocol is approximately 35 minutes. An operator must be present to transfer the hybridization plate from the thermal cycler when prompted by VWorks as shown in **step 17** below (<5 minutes after starting the protocol).

If the temperature of Bravo deck position 4 was not pre-set to 66°C, the protocol will pause while position 4 reaches temperature.

CAUTION It is important to complete **step 17** 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 NGS Bravo is completely prepared, with deck platforms at temperature and all components in place, before you transfer the sample plate to the Bravo deck.

17 When prompted by VWorks as shown below, quickly remove the PCR plate, containing the hybridization reactions held at 65°C, from the thermal cycler. Unseal the plate carefully to avoid

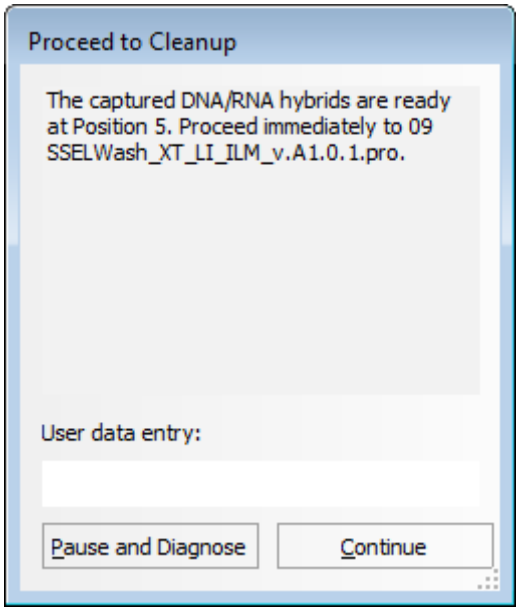
splashing, and quickly transfer the plate to position 4 of the Bravo deck, seated in the red insert. Click **Continue** to resume the protocol.



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

Use caution when handling components that contact heated deck positions.

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



Step 4. Wash the captured DNA

This step uses automation protocol **09 SSELWash_XT_LI_ILM** to automate washing of the captured DNA-RNA hybrids.

This step uses the component listed in **Table 53** in addition to components retained from the previous automation protocol.

Table 53 Reagents for capture wash protocol

Kit Component	Storage Location	Where Used
SureSelect Wash Buffer 1	SureSelect Target Enrichment Kit ILM Hyb Module, Box 1 (Post PCR), RT	**page 72**

Prepare the NGS Bravo and source plates

1. Retain the hybrid-capture bead suspension plate at position 5, the pre-warmed Wash Buffer 2 source plate at position 6, and the red PCR plate insert (empty) at position 4 of the Bravo deck. Clear the remaining Bravo deck positions of all plates and tip boxes.
2. Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in **Table 39**. See **page 19** for more information on how to do this step.

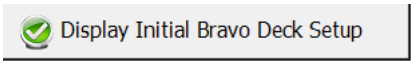
Table 54 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
4	76°C	Inheco Multi TEC control touchscreen (CPAC 2-1)
6	82°C	Inheco Multi TEC control touchscreen (CPAC 2-2)

3. Prepare an Eppendorf source plate labeled *Wash #1*. For each well to be processed, add 160 µL of SureSelect Wash Buffer 1. Place the Wash #1 source plate at position 3 of the Bravo deck.
4. Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water and place the reservoir at position 9 of the Bravo deck.

Setup and Run VWorks protocol 09 SSELWash_XT_LI_ILM

5. On the SureSelect setup form, under **Select Protocol**, select **09 SSELWash_XT_LI_ILM**.
6. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
7. Click **Display Initial Bravo Deck Setup**.



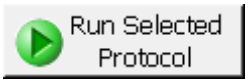
8 Verify that the Bravo deck has been set up according to the **Bravo Deck Setup** region of the form and as shown in **Table 55**.

Table 55 Initial Bravo deck configuration for 09 SSELWash_XT_LI_ILM

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
2	New tip box
3	*Wash #*1 Eppendorf source plate
4	**Empty** red insert
5	DNA-RNA hybrids captured on streptavidin beads in DeepWell plate
6	*Wash #2* DeepWell source plate seated on silver Nunc DeepWell insert (pre-heated during Capture protocol)
7	–(empty)–
8	Empty tip box
9	Nuclease-free water reservoir

9 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

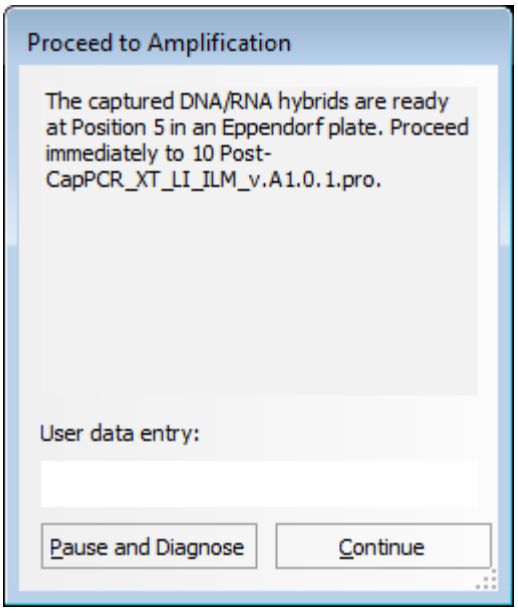
10 When setup and verification is complete, click **Run Selected Protocol**.



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

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

Remove the DNA sample plate from position 5 and seal the wells using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Store the plate on ice until it is used on **page 79**. Proceed immediately to the **10 Post-CapPCR_XT_LI_ILM** protocol, starting on **page 76**.



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 PCR-amplify, purify, assess quality and quantity of the libraries. Guidelines are provided for pooling the indexed samples for multiplexed sequencing and downstream sequencing steps.

Step 1. Amplify the captured DNA libraries

In this step, the Agilent NGS Bravo completes the liquid handling steps for PCR-amplification of the SureSelect-enriched DNA samples. After the PCR plate is prepared by the Agilent NGS Bravo, 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 59** on page 77 for cycle number recommendations.

This step uses the components listed in **Table 56**. Before you begin, thaw the reagents listed below and keep on ice. Before use, mix each component as directed.

Table 56 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 77**
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 77**
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 77**
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 77**

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

Prepare the NGS Bravo

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

2 Pre-set the temperature of Bravo deck positions 6 and 9 as indicated in **Table 57**. See **page 19** to **page 20** for more information on how to do this step.

Table 57 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
6	4°C	Inheco Multi TEC control touchscreen (CPAC 2-2)
9	0°C	ThermoCube control panel

3 Place a red PCR plate insert at Bravo deck position 6 and a silver deep well plate insert at Bravo deck position 9.

Pre-program the thermal cycler

4 Pre-program a thermal cycler (with the heated lid ON) with the program in **Table 58**. Start the program, then immediately pause the program, allowing the heated lid to reach temperature while you set up the reactions.

Table 58 Post-capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	9 to 14 See **Table 59** 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 59 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

5 Prepare the appropriate volume of post-capture PCR Master Mix, according to **Table 60**. Mix well using a vortex mixer and keep on ice..

Table 60 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

Table 60 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
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**

6 Using a fresh Nunc DeepWell plate, prepare the master mix source plate by adding the volume of PCR master mix indicated in **Table 61** to all wells of column 1 of the DeepWell plate.

Table 61 Preparation of the Master Mix Source Plate for 10 Post-CapPCR_XT_LI_ILM

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

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

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

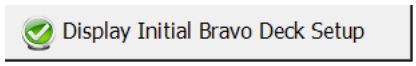
Setup and run VWorks protocol 10 Post-CapPCR_XT_LI_ILM

9 On the SureSelect setup form, under **Select Protocol to Run**, select **10 Post-CapPCR_XT_LI_ILM**.

10 Under **Select PCR plate Labware**, select the specific type of PCR plate used at position 6 of the Bravo deck.

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

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



13 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in **Table 62**.

Table 62 Initial Bravo deck configuration for 10 Post-CapPCR_XT_LI_ILM

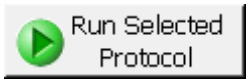
Location	Content
1	–(empty)–
2	New tip box
3	Captured DNA bead suspensions in Eppendorf twin.tec plate
4	–(empty)–

Table 62 Initial Bravo deck configuration for 10 Post-CapPCR_XT_LI_ILM

Location	Content
5	–(empty)–
6	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
7	–(empty)–
8	Empty tip box
9	Master mix plate containing PCR Master Mix in Column 1 (unsealed) seated on silver Nunc DeepWell insert

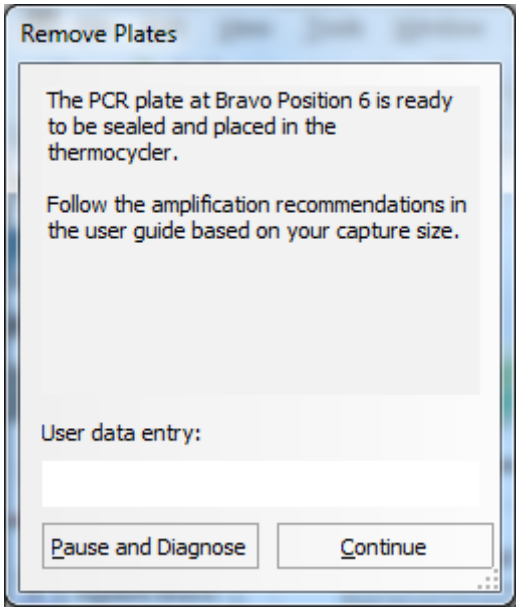
14 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

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



16 Running the **10 Post-CapPCR_XT_LI_ILM**, protocol takes approximately 10 minutes. Once complete, the PCR-ready samples, are located in the PCR plate at position 6 of the Bravo deck.

17 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.



18 Place the plate in the thermal cycler. Resume the thermal cycling program in **Table 58** on page 77.

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

Step 2. Purify the amplified libraries using AMPure XP beads

In this step, the Agilent NGS Bravo combines AMPure XP beads with the amplified target-enriched DNA and then collects and washes the bead-bound DNA.

This step uses protocol **11 Cleanup_Post-CapPCR_XT_LI_ILM**.

Prepare the NGS Bravo and reagents

1. Verify that the AMPure XP bead suspension is at room temperature. (If necessary, allow the bead solution to come to room temperature for at least 30 minutes.) *Do not freeze the beads at any time*.
2. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
3. Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in **Table 63**. See **page 19** for more information on how to do this step.

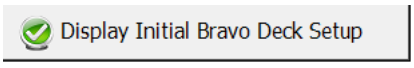
Table 63 Bravo Deck Temperature Presets

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

4. Place a red PCR plate insert at Bravo deck position 6.
5. Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.
6. Prepare a Nunc DeepWell source plate for the beads by adding 50 µL of homogeneous AMPure XP beads per well, for each well to be processed.
7. Prepare a Thermo Scientific Matrix reservoir containing 20 mL of nuclease-free water.
8. Prepare a separate Thermo Scientific Matrix reservoir containing 45 mL of freshly-prepared 70% ethanol.
9. Centrifuge the amplified DNA sample plate for 30 seconds to drive the well contents off the walls and plate seal.

Setup and run VWorks 11 Cleanup_Post-CapPCR_XT_LI_ILM

10. On the SureSelect setup form, under **Select Protocol**, select **11 Cleanup_Post-CapPCR_XT_LI_ILM**.
11. Under **Select PCR plate Labware**, select the specific type of PCR plate used for post-capture amplification.
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 Bravo Deck Setup**.



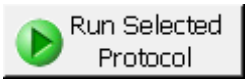
14. Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in **Table 64**.

Table 64 Initial Bravo deck setup for 11 Cleanup_Post-CapPCR_XT_LI_ILM

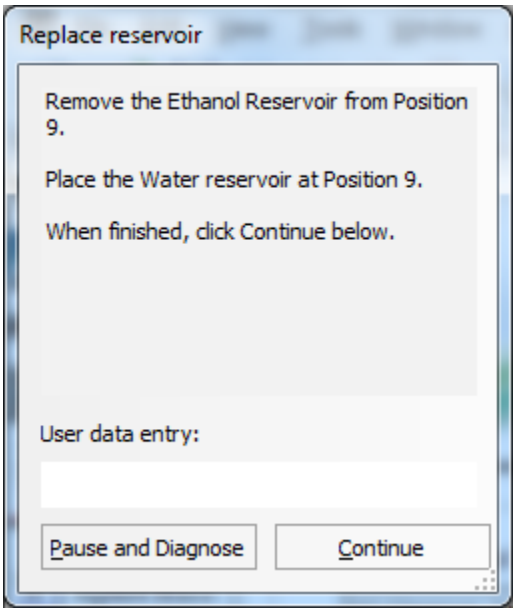
Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
2	New tip box
3	Empty Eppendorf plate
4	–(empty)–
5	AMPure XP beads in Nunc DeepWell plate (50 µL beads per processing well)
6	Amplified DNA libraries in unsealed PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
7	–(empty)–
8	Empty tip box
9	70% ethanol in Matrix reservoir

15 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

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



Running the **11 Cleanup_Post-CapPCR_XT_LI_ILM** protocol takes approximately 45 minutes. An operator must be present during the run to complete tip box replacement and to transfer the water reservoir to the Bravo deck, when directed by the VWorks prompt shown below.



Once the protocol is complete, the purified DNA samples are located in the Eppendorf plate at position 3 of the Bravo deck.

Step 3. Assess sequencing library DNA quantity and quality

Sample analysis can be completed using one of two options.

- Option 1: Prepare the analytical plate using automation (protocol **12 TS_HighSensitivity_D1000**) and preform analysis on the Agilent 4200 TapeStation. See **"Option 1: Analysis using an Agilent 4200 TapeStation Instrument and High Sensitivity D1000 ScreenTape"** on page 82.
- 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 86.

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

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

This section describes use of automation protocol **12 TS_HighSensitivity_D1000** to prepare samples for analysis. The automation protocol prepares the assay sample plate by combining 2 µL of each DNA sample with 2 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.

NOTE

If desired, the assay sample plate may also be prepared using manual liquid-handling steps as described in the High Sensitivity D1000 Assay Quick Guide above and then following the electropherogram analysis guidelines on **page 85**.

Prepare the NGS Bravo and Sample Buffer source plate

1. Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
2. Pre-set the temperature of Bravo deck position 4 to 4°C using the Inheco Multi TEC control touchscreen, as described in **Setting the Temperature of Bravo Deck Heat Blocks**. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
3. Turn off the ThermoCube device (see **page 20**) to restore position 9 of the Bravo deck to room temperature.
4. Using the same Eppendorf twin.tec plate that was used for the 07 Hyb_XT_LI_ILM run, prepare the Sample Buffer source plate at room temperature. Add the volume of High Sensitivity D1000 Sample Buffer indicated in **Table 65** to each well of column 4 of the plate. The final configuration of the sample buffer source plate is shown in **Figure 10**.

Table 65 Preparation of the Sample Buffer Source Plate for 12 TS_HighSensitivity_D1000

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)	7.0 µL	9.0 µL	11.0 µL	13.0 µL	17.0 µL	29.0 µL

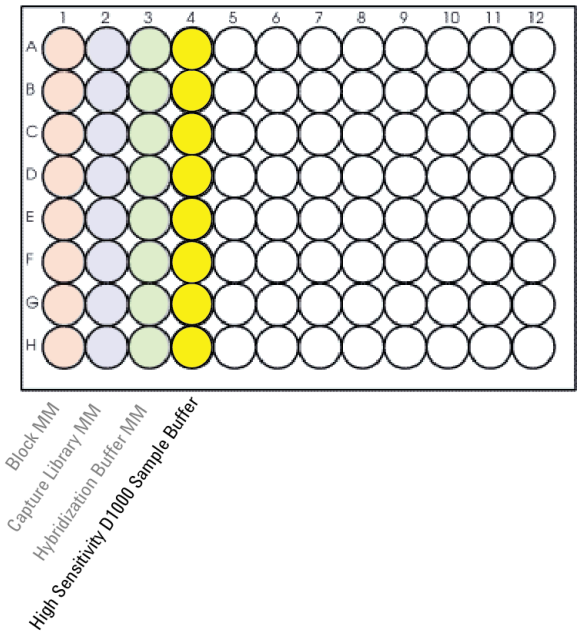


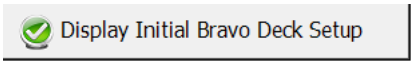
Figure 10 Configuration of the sample buffer source plate for **12 TS_HighSensitivity_D1000**. Columns 1-3 were used to dispense master mixes during the hybridization protocol.

Setup and Run VWorks protocol 12 TS_HighSensitivity_D1000

5 On the SureSelect setup form, under **Select Protocol to Run**, select **12 TS_HighSensitivity_D1000**.

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

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



8 Load the Bravo deck according to the Bravo Deck Setup region of the form and as shown in Table 66.

Table 66 Initial Bravo deck configuration for 12 TS_HighSensitivity_D1000

Location	Content
1	–(empty)–
2	New tip box
3	–(empty)–
4	Amplified post-capture libraries in Eppendorf twin.tec plate (unsealed)
5	–(empty)–
6	Empty Eppendorf twin.tec plate
7	–(empty)–

Table 66 Initial Bravo deck configuration for 12 TS_HighSensitivity_D1000

Location	Content
8	Empty tip box
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 Bravo, 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.

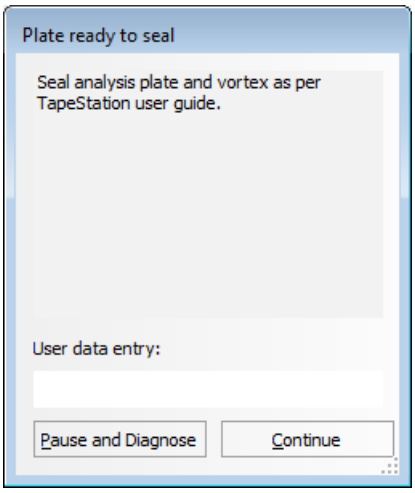
9 Verify that the **Current Tip State** indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See **page 22** for more information on using this segment of the form during the run.

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

Running the 12 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 87**.

11 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

12 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.

13 Verify that the electropherogram shows the peak of DNA fragment size positioned between 200 and 400 bp. Sample electropherograms are shown in **Figure 11** (library prepared from high-quality DNA), **Figure 12** (library prepared from medium-quality FFPE DNA), and **Figure 13** (library prepared from low-quality FFPE DNA).

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

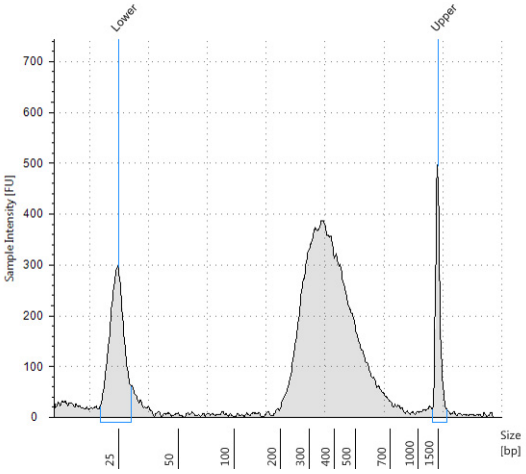


Figure 11 Post-capture library prepared from a high-quality gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

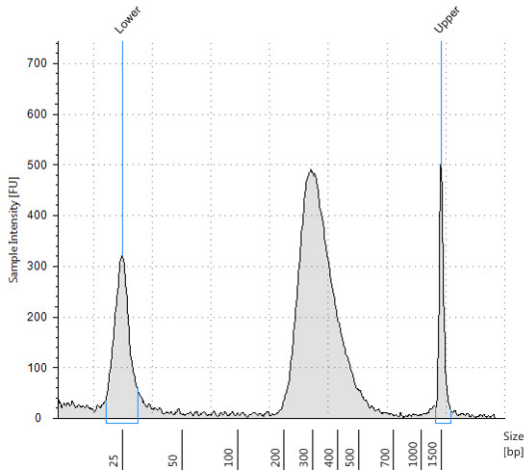


Figure 12 Post-capture library prepared from a typical FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

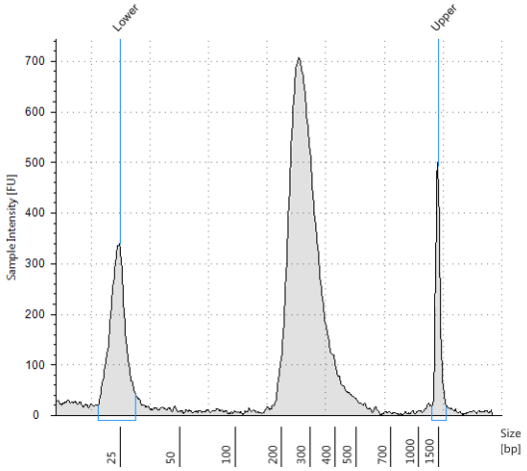


Figure 13 Post-capture library prepared from a low-quality FFPE gDNA sample analyzed using a High Sensitivity D1000 ScreenTape assay.

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 11** through **Figure 13**). **Table 67** includes links to assay instructions.

Table 67 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 plate 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 that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for your research design. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform and the amount of sequencing data required per sample.

Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool using one of the 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 68 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 68 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 14**.

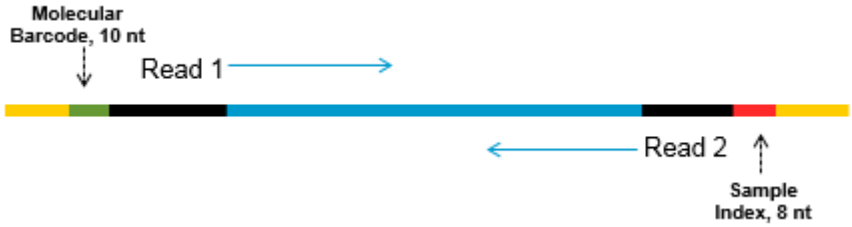


Figure 14 Content of SureSelect XT Low Input sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the sample index (red), the molecular barcode (green) and the library bridge PCR primers (yellow).

Libraries can be sequenced on the Illumina HiSeq, MiSeq, NextSeq, or NovaSeq platform using the run type and chemistry combinations shown in **Table 69**.

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. See **Table 69** for alternative run mode/chemistry options for the HiSeq2500 platform. 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.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See **Table 69** 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 69** 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 69**.

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

Table 69 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 or v1.5	300–600 pM
NovaSeq 6000	Xp Workflow Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1 or v1.5	200–400 pM

* 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.

† Do not use HiSeq 2500 High Output (v4 chemistry) runs if your analysis pipeline includes molecular barcode (i5) reads. Reduced molecular barcode sequence quality and lowered Q scores have been observed in sequences obtained from HiSeq 2500 platform runs under these conditions. Sequencing performance for Read 1, Read 2, and sample-level index (i7) reads is not affected.

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 82** on page 106 and **Table 83** on page 107.

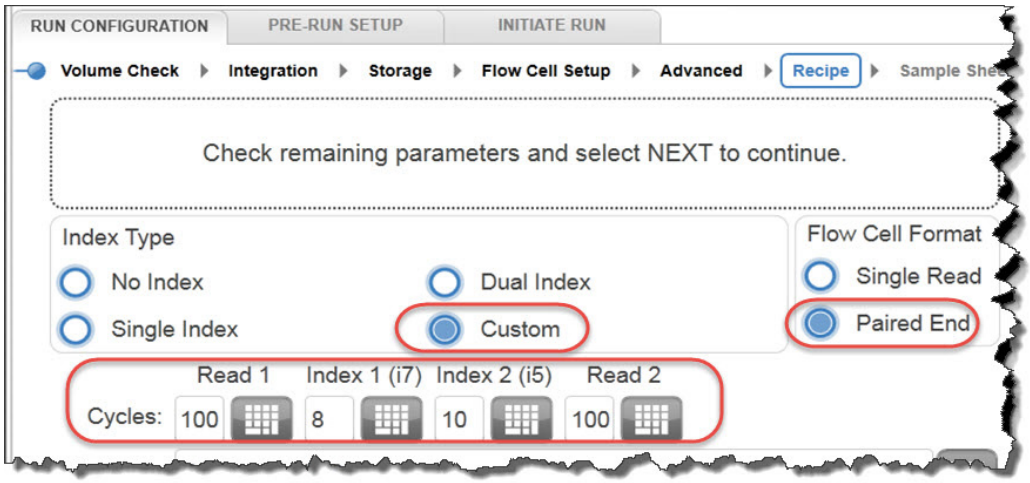
CAUTION The 8-bp index sequences in SureSelect XT Low Input Index Primers 1-96 and 97-192 differ from the 8-bp index sequences in index primers A01 through H12 in Agilent's SureSelect XT system.

- Optional use of the degenerate molecular barcode (i5) requires a 10-bp index read.
- For the HiSeq, NextSeq, and NovaSeq platforms, set up the run using the instrument's user interface, following the guidelines on **page 91**.
- For the MiSeq platform, set up the run using Illumina Experiment Manager (IEM) using the steps detailed on **page 92** to **page 94** to generate a custom sample sheet.
- 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.
- Demultiplex using the appropriate Illumina software to generate paired-end reads in FASTQ format. If your workflow excludes the use of molecular barcodes, you can demultiplex using Illumina's bcl2fastq, BCL Convert, or DRAGEN software. If your workflow includes the use of molecular barcodes, demultiplex using Illumina's bcl2fastq software, using the I2 molecular barcode retrieval steps described below.

- Retrieval of I2 index files containing the molecular barcode (i5) index reads requires offline conversion of *.bcl* to *.fastq* files. For information on how to do this step, see **page 91** for HiSeq, NextSeq, and NovaSeq runs and see **page 94** for MiSeq runs.
- 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 95** 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 96** for more information).

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 with molecular barcode (i5) data collection 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>
```

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 82** on page 106 and **Table 83** on page 107 for sequences of the SureSelect XT Low Input system indexes.

If your workflow excludes the use of molecular barcodes, modify the steps in this section to collect a Single Index Read (i7) and omit steps for I2 molecular barcode retrieval.

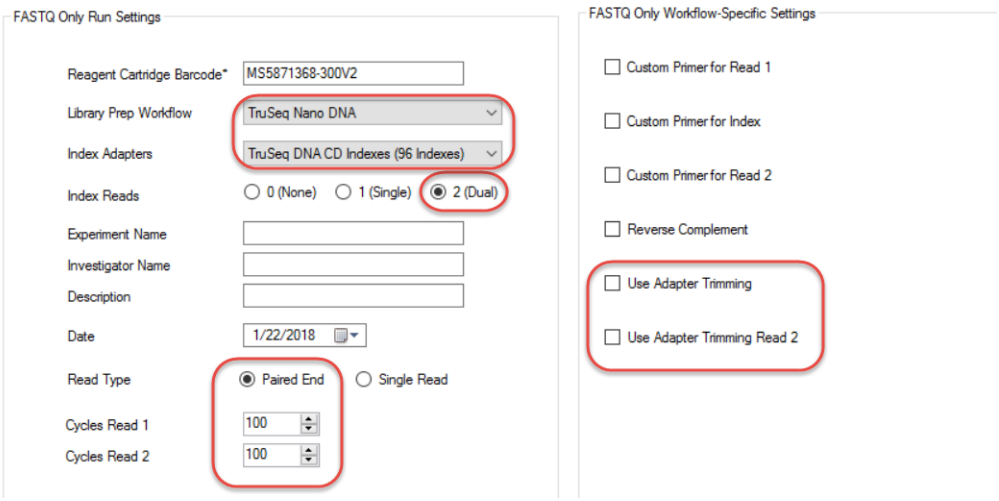
Set up a custom Sample Sheet:

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

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

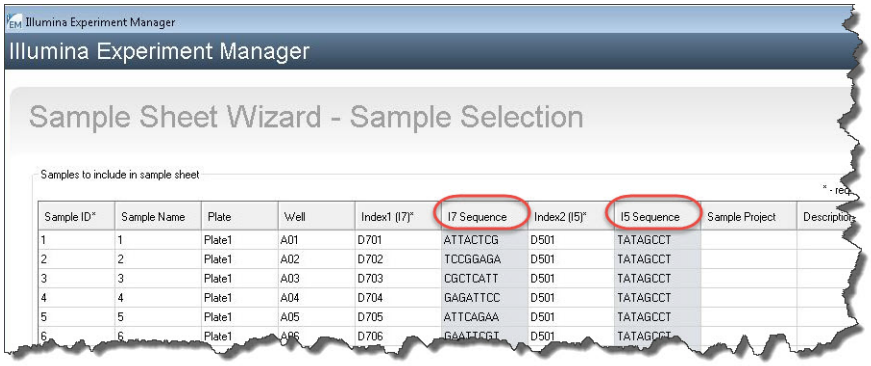
2 On the **Workflow Parameters** screen, enter the run information, making sure to specify the key parameters highlighted below. In the *Library Prep Workflow* field, select **TruSeq Nano DNA**. In the *Index Adapters* field, select **TruSeq DNA CD Indexes (96 Indexes)**. Make sure to clear both adaptor-trimming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

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



3 Using the Sample Sheet Wizard, set up a New Plate, entering the required information for each sample to be sequenced. In the **I7 Sequence** column, assign each sample to any of the Illumina i7 indexes. The index will be corrected to 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 15**).

- 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 82** on page 106 and **Table 83** on page 107 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 94**. Sample sheets processed offline using Illumina's bcl2fastq software must not contain N_{10} wildcard index sequences. See **page 92** for more information.

[Header]									
IEMFileVer	5								
Experimen	XTHS								
Date	1/22/2018								
Workflow	GenerateFASTQ								
Applicatio	FASTQ Only								
Instrumen	MiSeq								
Assay	TruSeq Nano DNA								
Index Ada	TruSeq DNA CD Indexes (96 Indexes)								
Description									
Chemistry	Amplicon								
[Reads]									
100									
100									
[Settings]									
ReverseCo	0								
[Data]									
Sample_ID	Sample_Name	Sample_Plate	Sample_W	Index_Plate_W	I7_Index_ID	index	I5_Index_I	index2	Sample
XTHS-S1	XTHS-S1	1	A01	A01	A01	GTCTGTCA	MBC	NNNNNNNNNN	XTHS
XTHS-S2	XTHS-S2	1	B01	B01	B01	TGAAGAGA	MBC	NNNNNNNNNN	XTHS

Figure 15 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 91** for HiSeq and NextSeq platform runs may also be applied to MiSeq platform runs.

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 90** for guidelines). The demultiplexed FASTQ data needs to be pre-processed to remove sequencing adaptors and utilize the i5 molecular barcode 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 70**.

Table 70 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 30**	Qualification of DNA Integrity	Not required	Required
DNA input for Library Preparation **page 31**	Input amount and means of quantification	10 ng to 200 ng, quantified by Qubit assay	Based on determined DNA integrity (see **Table 11** on page 31 and **Table 12** on page 31)
DNA Shearing **page 32**	Mode of DNA Shearing	2 × 120 seconds	240 seconds (continuous)
Pre-capture PCR **page 42**	Cycle number	8–11	11–14
Sequencing **page 100**	Output augmentation	Per project requirements	1× to 10× based on determined DNA integrity (see **Table 71** and **Table 72** on page 100)

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 71**. 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 71 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 72**. 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 72 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 and reference information for use during the downstream sample sequencing steps.

Kit Contents

SureSelect XT Low Input Reagent Kits include the component kits listed in **Table 73** or **Table 74**. Detailed contents of each of the multi-part component kits are shown in **Table 75** through **Table 78**.

Table 73 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 74 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 75 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 76 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 80** on page 105 for a plate map and see **Table 82** on page 106 for index sequences.

† See **Table 81** on page 105 for a plate map and see **Table 83** on page 107 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 77 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 78 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 79**. The SureSelect XT Low Input Reagent Kits included in these bundles are supplied with the same component kits listed in **Table 73** or **Table 74** on **page 102**.

Table 79 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.)

Table 80 Plate map for SureSelect XT Low Input 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 81 Plate map for SureSelect XT Low Input 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

Nucleotide Sequences of SureSelect XT Low Input Indexes

Each index is 8 NT in length. See **page 90** for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 82 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 83 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

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 39** for library preparation purification and on **page 47** for pre-capture purification. 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 52** to **page 53**. 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 automation protocol 04 Cleanup_Pre-CapPCR_XT_LI_ILM.

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 61**, 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 80**.

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

- ✔ Minimize the amount of time that hybridization reactions are exposed to RT conditions during hybridization setup. Locate a thermal cycler in close proximity to the Bravo NGS Bravo to retain the 65°C sample temperature during transfer step (**step 24** on **page 67**).

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 38** on page 59).

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In This Book

This guide contains information to run the SureSelect^{XT} Low Input Automated Library Prep and Target Enrichment protocol using Agilent's NGS Bravo Option A.

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