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

Automated using Agilent NGS Workstation Option B

Protocol

Version N0, November 2020

SureSelect platform manufactured with Agilent SurePrint Technology

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

Agilent Technologies

Notices

Manual Part Number

G7550-90000

Edition

Version N0, November 2020

Printed in USA

Agilent Technologies, Inc.
5301 Stevens Creek Blvd
Santa Clara, CA 95051 USA

Acknowledgment

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Or send an e-mail to:
ngs.support@agilent.com

For all other regions

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CAUTION

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In this Guide...

This guide describes an optimized protocol for Illumina paired-end multiplexed library preparation using the Agilent SureSelect^{XT} Automated Library Prep and Capture System.

This protocol is specifically developed and optimized to capture the genomic regions of interest using Agilent's SureSelect system to enrich targeted regions of the genome from repetitive sequences and sequences unrelated to the research focus prior to sample sequencing. Sample processing steps are automated using the Agilent NGS Workstation Option B.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Using the Agilent NGS Workstation for SureSelect Target Enrichment

This chapter contains an orientation to the Agilent NGS Workstation, an overview of the SureSelect target enrichment protocol, and considerations for designing SureSelect experiments for automated processing using the Agilent NGS Workstation.

3 Sample Preparation (3 µg DNA Samples)

This chapter describes the steps to prepare the DNA samples for target enrichment when starting with 3 µg of gDNA.

4 Sample Preparation (200 ng DNA Samples)

This chapter describes the steps to prepare the DNA samples for target enrichment when starting with 200 ng of gDNA.

5 Hybridization

This chapter describes the steps to hybridize and capture samples.

6 Indexing

This chapter describes the steps to amplify, purify, and assess quality of the sample libraries. Samples are pooled by mass prior to sequencing.

7 Reference

This chapter contains reference information.

What's New in Version N0

- Updates to thermal cycler recommendations (see Table 5 on page 15).

What's New in Version M0

- Support for revised SureSelect custom probe products, produced using an updated manufacturing process beginning August, 2020 (see Table 3 on page 14). Custom probes produced using the legacy manufacturing process are also fully supported by the protocols in this document. Probe information was reorganized (see Table 2 on page 13 through Table 4 on page 14), and probe nomenclature throughout document was updated.
- Updates to ordering information for Dynabeads MyOne Streptavidin T1 beads, 1X Low TE Buffer, and AMPure XP Kits (Table 1 on page 12) and for Qubit Fluorometer (Table 5 on page 15).
- Updates to Agilent TapeStation 4200/4150 ordering information (Table 5 on page 17) and sample mixing information (for example, see *Caution* on page 68).
- Support for 5200 Fragment Analyzer (see footnote to Table 5 on page 17).
- Minor updates to DNA shearing set up instructions (see page 38 and page 70).
- Updates to organization of "Kit Contents" on page 150.
- Updates to Technical Support contact information (see page 2)

What's New in Version L0

- Updates to sequencing guidelines including support for use of Illumina's NovaSeq platform and minor revisions to guidelines for Illumina kit selection, seeding concentrations, and run setup for various platforms (see page 146 to page 147)

- Updates to Probe Capture Library selection tables (see Table 2 on page 13 through Table 4 on page 14) to show current product offerings in a simplified format
- Updates to ordering information for materials purchased from Thermo Fisher Scientific (see Table 1 on page 12 and Table 5 on page 15)
- Update to step 2 on page 38 and on page 70 to include formulation of Low TE Buffer
- Update to *Note* on page 38 and page 70 to support use of alternative shearing parameters
- Minor updates to 2100 Bioanalyzer and 4200 TapeStation use instructions and reference document links (see page 45, page 46, page 66, page 68 page 140, and page 142)
- Support for 4150 TapeStation (see footnote to Table 5 on page 17)
- Update to post-capture PCR protocol to remove plate spin step immediately before on-bead PCR (see page 134)
- Update to "Technical Support" contact information

What's New in Version K0

- Support for VWorks software version 13.1.0.1366 and Agilent NGS Workstation Option B p/n G5574AA (see Table 5 on page 15)
- Updates to downstream sequencing instructions including sequencing kit selection and seeding concentration guidelines (see page 146)
- Updates to Agilent 2100 Bioanalyzer system ordering information (see Table 5 on page 17)
- Addition of Agilent 4200 TapeStation system-compatible plasticware ordering information (see Table 5 on page 17)
- Updates to reference information for Agilent NGS Workstation component user guides (see Table 6 on page 20)

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1 Before You Begin

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

CAUTION

This Protocol supports the SureSelect Target Enrichment workflow with on-bead post-capture PCR, using version 1.5.1 (**v1.5.1)** VWorks SureSelect automation protocols.

If your VWorks SureSelect setup form displays earlier versions of the automation protocols, please contact service.automation@agilent.com for assistance.

NOTE

This protocol describes automated sample processing using the Agilent NGS Workstation. For non-automated sample processing procedures for Agilent's SureSelect^{XT} Target Enrichment Kit for Illumina Multiplex Sequencing, see publication G7530-90000.

NOTE

This protocol differs from other SureSelect protocols at several steps. Pay close attention to the primers used for each amplification step and the blocking agents used during hybridization.

Procedural Notes

- This User Guide includes protocols for library preparation using either 3 µg DNA samples (see Chapter 3 on page 37) or 200 ng DNA samples (see Chapter 4 on page 69). Make sure that you are following the appropriate protocol for your DNA input amount. After the prepared libraries are amplified, both DNA input options use the same protocol for hybridization and post-capture processing.
- Certain protocol steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the Agilent NGS Workstation to allow rapid and efficient plate transfer.
- Prepare and load the Agilent NGS Workstation as detailed in each of the protocol steps before initiating each automated protocol run. When loading plates in the workstation's Labware MiniHub, always place plates in the orientation shown in Figure 3 on page 42.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Do not mix stock solutions and reactions containing gDNA on a vortex mixer. Instead, gently tap the tube with your finger to mix the sample.
- Avoid repeated freeze-thaw cycles of stock and diluted gDNA solutions. Possible stopping points, where gDNA samples may be stored overnight at 4°C, are marked in the protocol. When storing samples for >24 hours, store the samples at –20°C, but do not subject the samples to multiple freeze/thaw cycles.
- When preparing frozen reagent stock solutions for use:
  1. Thaw the aliquot as rapidly as possible without heating above room temperature.
  2. Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
  3. Store on ice or in a cold block until use.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION

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

Required Reagents

Table 1 Required Reagents

Description	Vendor and part number
SureSelect, ClearSeq or OneSeq Probe Capture Library	Select the appropriate probe from Table 2, Table 3 or Table 4
SureSelect^{XT} Automation Reagent Kit*	
HiSeq platform (HSQ), 96 reactions	Agilent p/n G9641B
HiSeq platform (HSQ), 480 reactions	Agilent p/n G9641C
MiSeq platform (MSQ), 96 reactions	Agilent p/n G9642B
MiSeq platform (MSQ), 480 reactions	Agilent p/n G9642C
Herculase II Fusion DNA Polymerase, 400 reactions (includes dNTP mix and 5x Buffer)	Agilent p/n 600679
QPCR NGS Library Quantification Kit (Illumina GA)	Agilent p/n G4880A
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930
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
AMPure XP Kit	Beckman Coulter Genomics
60 mL	p/n A63881
450 mL	p/n A63882
Qubit BR dsDNA Assay Kit	Thermo Fisher Scientific
100 assays, 2-1000 ng	p/n Q32850
500 assays, 2-1000 ng	p/n Q32853
Dynabeads MyOne Streptavidin T1	Thermo Fisher Scientific
2 mL	p/n 65601
10 mL	p/n 65602
50 mL	p/n 65604D
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023

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

Table 2 Compatible Pre-Designed Probes for Automation

Probe Capture Library	96 Reactions
SureSelect XT Human All Exon V7	5191-4006
SureSelect XT Human All Exon V6	5190-8865
SureSelect XT Human All Exon V6 + UTRs	5190-8883
SureSelect XT Human All Exon V6 + COSMIC	5190-9309
SureSelect XT Clinical Research Exome V2	5190-9493
SureSelect XT Focused Exome	5190-7789
SureSelect XT Mouse All Exon	5190-4643
ClearSeq Comprehensive Cancer XT	5190-8013
ClearSeq Inherited Disease XT	5190-7520
Pre-designed Probes customized with additional *Plus* custom content	
SureSelect XT 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.
SureSelect XT 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	
SureSelect XT Focused Exome Plus 1	
SureSelect XT Focused Exome Plus 2	
ClearSeq Comprehensive Cancer Plus XT	
ClearSeq Inherited Disease Plus XT	

Table 3 Compatible Custom Probes for Automation*

Probe Capture Library	96 Reactions	480 Reactions
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.	
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		

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

Table 4 Compatible OneSeq CNV Probes for Automation

Probe Capture Library	96 Reactions
OneSeq 300kb CNV Backbone + Human All Exon V7	5191-4024
OneSeq 1Mb CNV Backbone + Human All Exon V7	5191-4027
OneSeq Constitutional Research Panel	5190-8704
Pre-designed Probes customized with additional *Plus* custom content	
OneSeq 1Mb CNV Backbone + Custom 1–499 kb	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.
OneSeq 1Mb CNV Backbone + Custom 0.5–2.9 Mb	
OneSeq 1Mb CNV Backbone + Custom 3–5.9 Mb	
OneSeq 1Mb CNV Backbone + Custom 6–11.9 Mb	
OneSeq 1Mb CNV Backbone + Custom 12–24 Mb	
OneSeq Hi Res CNV Backbone + Custom 1–499 kb	
OneSeq Hi Res CNV Backbone + Custom 0.5 –2.9 Mb	
OneSeq Hi Res CNV Backbone + Custom 3–5.9 Mb	
OneSeq Hi Res CNV Backbone + Custom 6–11.9 Mb	

Required Equipment

Table 5 Required Equipment

Description	Vendor and part number
Agilent NGS Workstation Option B Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5522A (VWorks software version 13.1.0.1366, 13.0.0.1360, or 11.3.0.1195) OR Agilent p/n G5574AA (VWorks software version 13.1.0.1366)
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Thermal cycler and accessories	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 Fisher Scientific p/n 1064156
Nunc DeepWell Plates, sterile, 1.3-mL well volume	Thermo Fisher 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
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Qubit Fluorometer	Thermo Fisher Scientific p/n Q33238
Qubit assay tubes	Thermo Fisher Scientific p/n Q32856
Covaris Sample Preparation System, S-series or E-series model	Covaris

Table 5 Required Equipment (continued)

Description	Vendor and part number
Covaris sample holders	
96 microTUBE plate (E-series only)	Covaris p/n 520078
microTUBE for individual sample processing	Covaris p/n 520045
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Pipettes (10-, 20-, 200-, and 1000-µl capacity)	Rainin Pipet-Lite Pipettes or equivalent
Vacuum concentrator	Savant SpeedVac, model DNA120, with 96-well plate rotor, model RD2MP, or equivalent
Magnetic separator	DynaMag-50 magnet, Thermo Fisher Scientific p/n 123-02D or equivalent
Mx3005P Real-Time PCR System	Agilent p/n 401449 or equivalent
Mx3000P/Mx3005P 96-well tube plates	Agilent p/n 410088 or equivalent
Mx3000P/Mx3005P optical strip caps	Agilent p/n 401425 or equivalent
Nucleic acid surface decontamination wipes	DNA Away Surface Decontaminant Wipes, Thermo Scientific p/n 7008, or equivalent
Ice bucket	general laboratory supplier

Table 5 Required Equipment (continued)

Description	Vendor and part number
Powder-free gloves	general laboratory supplier
Sterile, nuclease-free aerosol barrier pipette tips	general laboratory supplier
Vortex mixer	general laboratory supplier
Timer	general laboratory supplier
DNA Analysis Platform and Consumables*	
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
OR	
Agilent 4200/4150 TapeStation	Agilent p/n G2991AA/G2992AA
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585

* DNA samples may also be analyzed using the Agilent 5200 Fragment Analyzer, p/n M5310AA, and associated NGS Fragment Kits (DNF-473-0500 and DNF-474-0500). Implement any sample dilution instructions provided in protocols in this document, and then follow the assay instructions provided for each NGS Fragment Kit.

2 Using the Agilent NGS Workstation for SureSelect Target Enrichment

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Overview of the SureSelect Target Enrichment Procedure 30
Experimental Setup Considerations for Automated Runs 33

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

About the Agilent NGS Workstation

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

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

Table 6 Agilent NGS Workstation components User Guide reference information

Device	User Guide part number
Bravo Platform	G5562-90000
VWorks Software	G5415-90068 (VWorks versions 13.1.0.1366 and 13.0.0.1360), or G5415-90063 (VWorks version 11.3.0.1195)
BenchCel Microplate Handler	G5400-90004
Labware MiniHub	G5471-90002
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 seven interchangeable fixed-tip or disposable-tip pipette heads, it accurately dispenses fluids from 0.1 µL to 250 µL.

Bravo Platform Deck

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

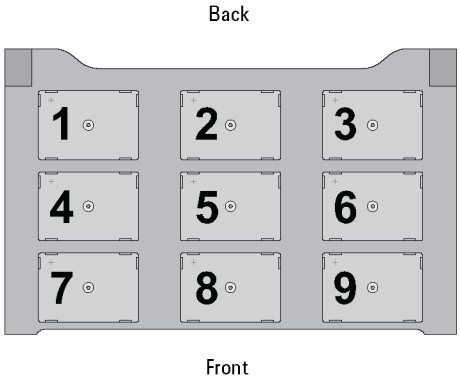


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

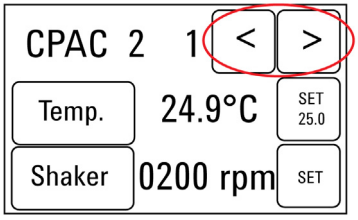
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

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

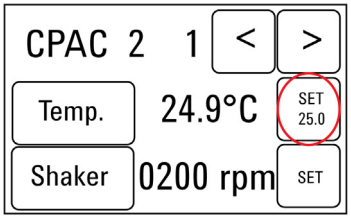
Table 7 Inheco Multi TEC Control touchscreen designations

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

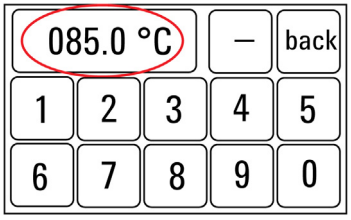
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



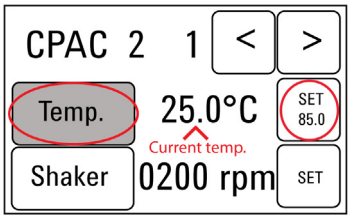
2 To set the temperature of the selected block, press the SET button.



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



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



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

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

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

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

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

3 Press the **START** button.

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

VWorks Automation Control Software

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

NOTE

The instructions in this manual are compatible with VWorks software version 13.1.0.1366, 13.0.0.1360 or 11.3.0.1195, including SureSelect^{XT} automation protocols version 1.5.1.

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

Logging in to the VWorks software

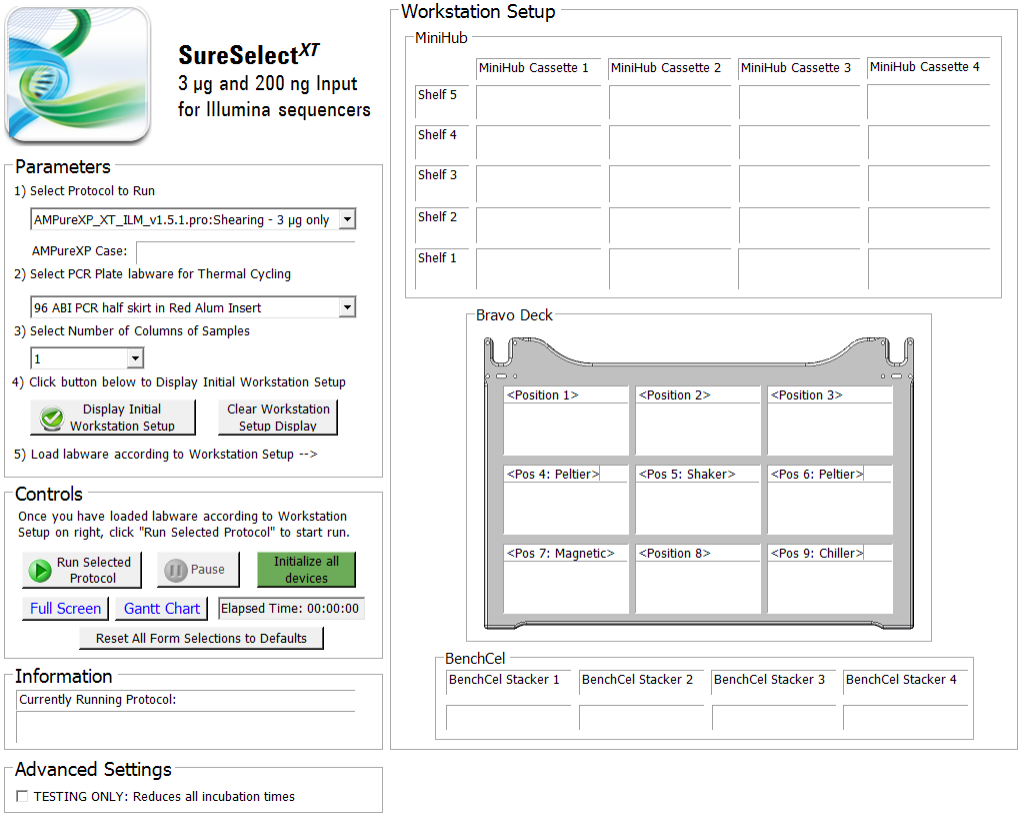
1. Double-click the VWorks icon or the XT_ILM_v1.5.1.VWForm shortcut on the Windows desktop to start the VWorks software.
2. If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
3. In the User Authentication dialog, type your VWorks user name and password, and click **OK**. (If no user account is set up, contact the administrator.)

VWorks protocol and runset files

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

Using the SureSelectXT_ILM_v1.5.1.VWForm to setup and start a run

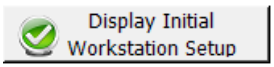
Use the VWorks form SureSelectXT_ILM_v1.5.1.VWForm, shown below, to set up and start each SureSelect automation protocol or runset.



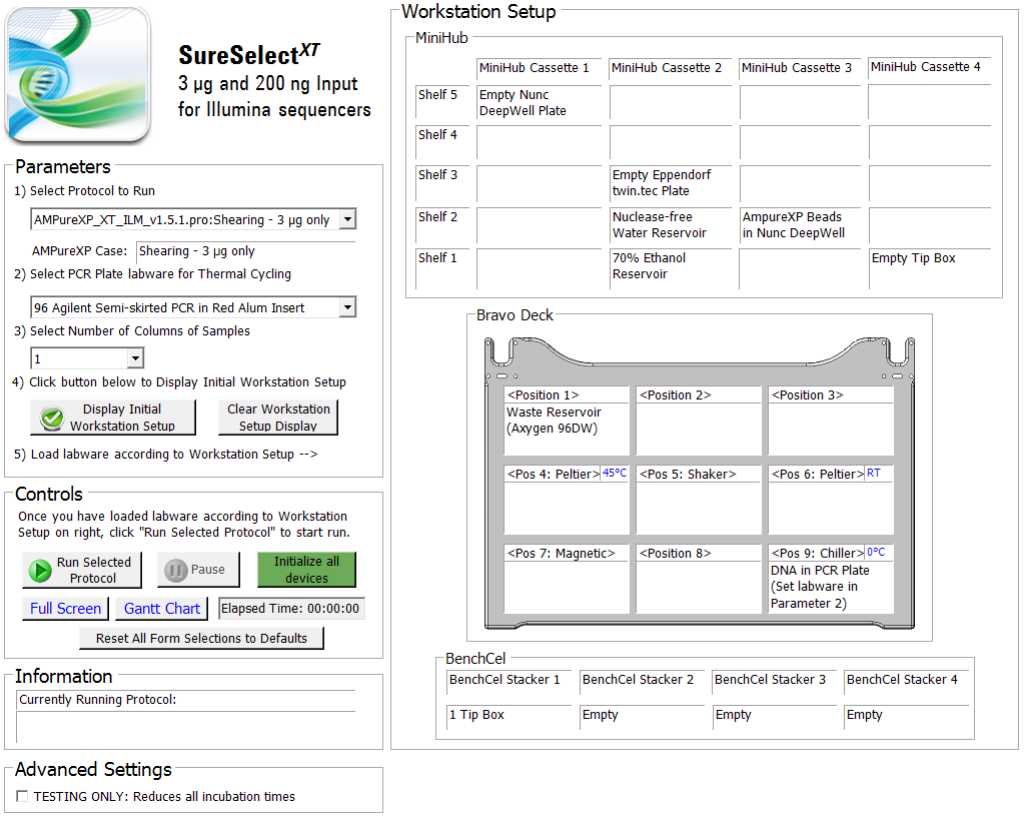
1 Open the form using the XT_ILM_v1.5.1.VWForm shortcut on your desktop.

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

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



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

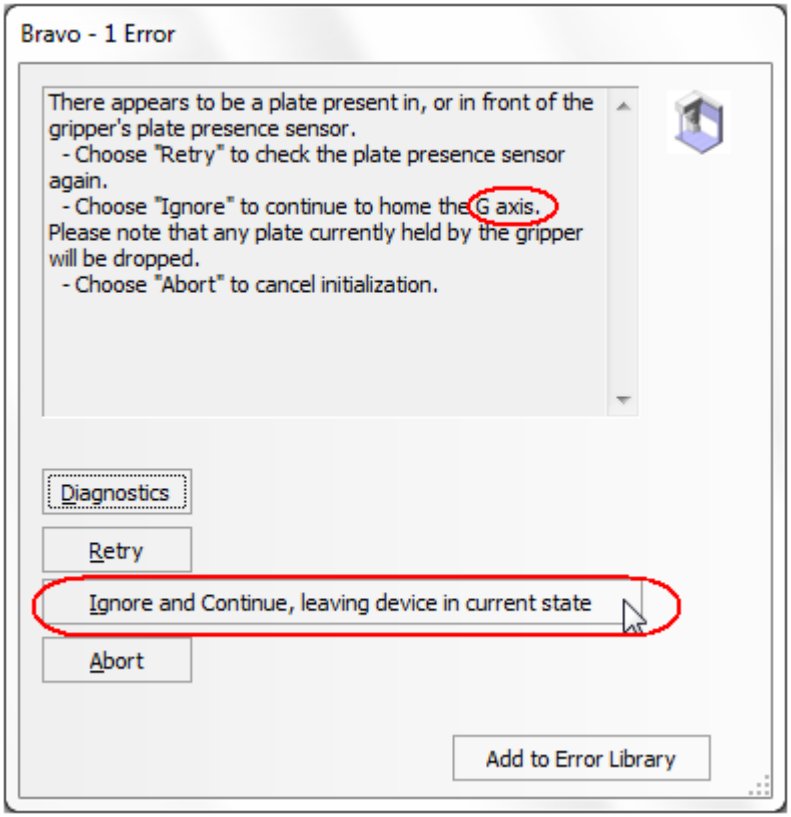


5 After verifying that the NGS Workstation has been set up correctly, click **Run Selected Protocol**.

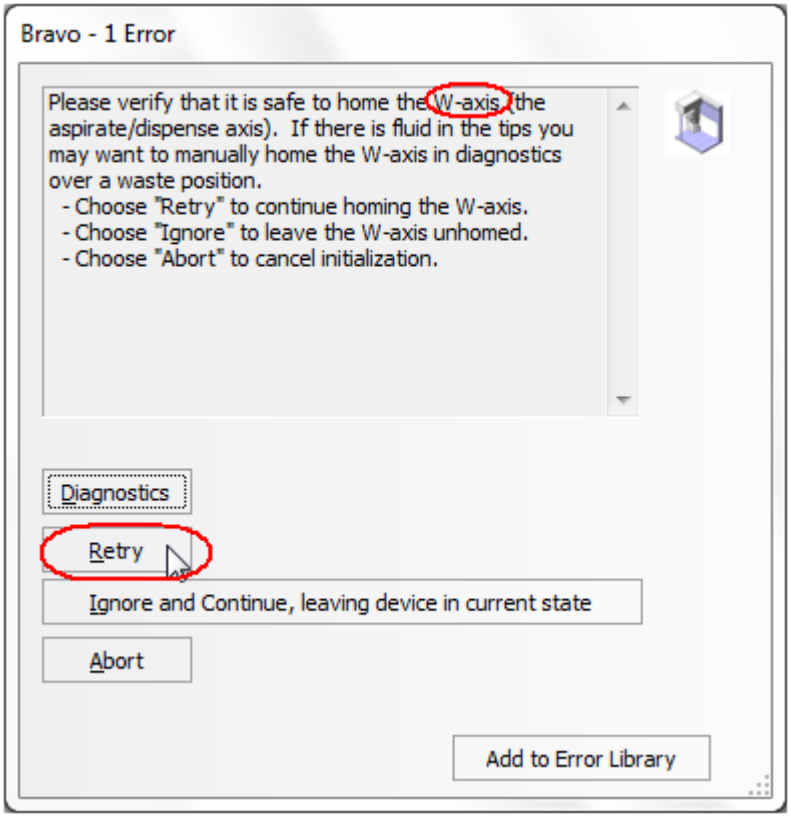
Error messages encountered at start of run

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

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



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



Verifying the Simulation setting

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

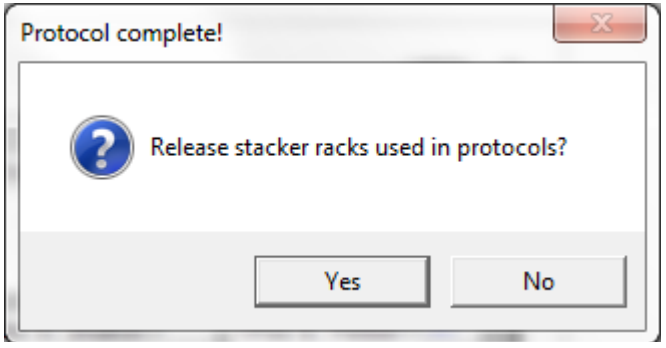
1 Verify that **Simulation is off** is displayed on the status indicator (accessible by clicking **View** > **Control Toolbar**).

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

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

Finishing a protocol or runset

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



Overview of the SureSelect Target Enrichment Procedure

Figure 2 summarizes the SureSelect 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. The samples are then tagged by PCR with an index sequence. Depending on the target size of the SureSelect capture, multiple samples can be pooled and sequenced in a single lane using the Illumina-specified multiplex index tags that are provided with SureSelect Library Prep kits.

The SureSelect^{XT} automated target enrichment system is compatible with gDNA samples containing either 3 µg or 200 ng DNA, with minor differences in the VWorks protocols used during the Sample Preparation segment of the workflow for the two DNA input options. Both DNA input options use identical automation protocols for the Hybridization and Indexing segments of the workflow.

When starting with 3 µg gDNA samples, see Table 8 for a summary of the VWorks protocols used during the workflow. Then, see Sample Preparation (3 µg DNA Samples), Hybridization, and Indexing chapters for complete instructions for use of the VWorks protocols for sample processing.

When starting with 200 ng gDNA samples, see Table 9 for a summary of the VWorks protocols used during the workflow. Then, see Sample Preparation (200 ng DNA Samples), Hybridization, and Indexing chapters for complete instructions for use of the VWorks protocols for sample processing.

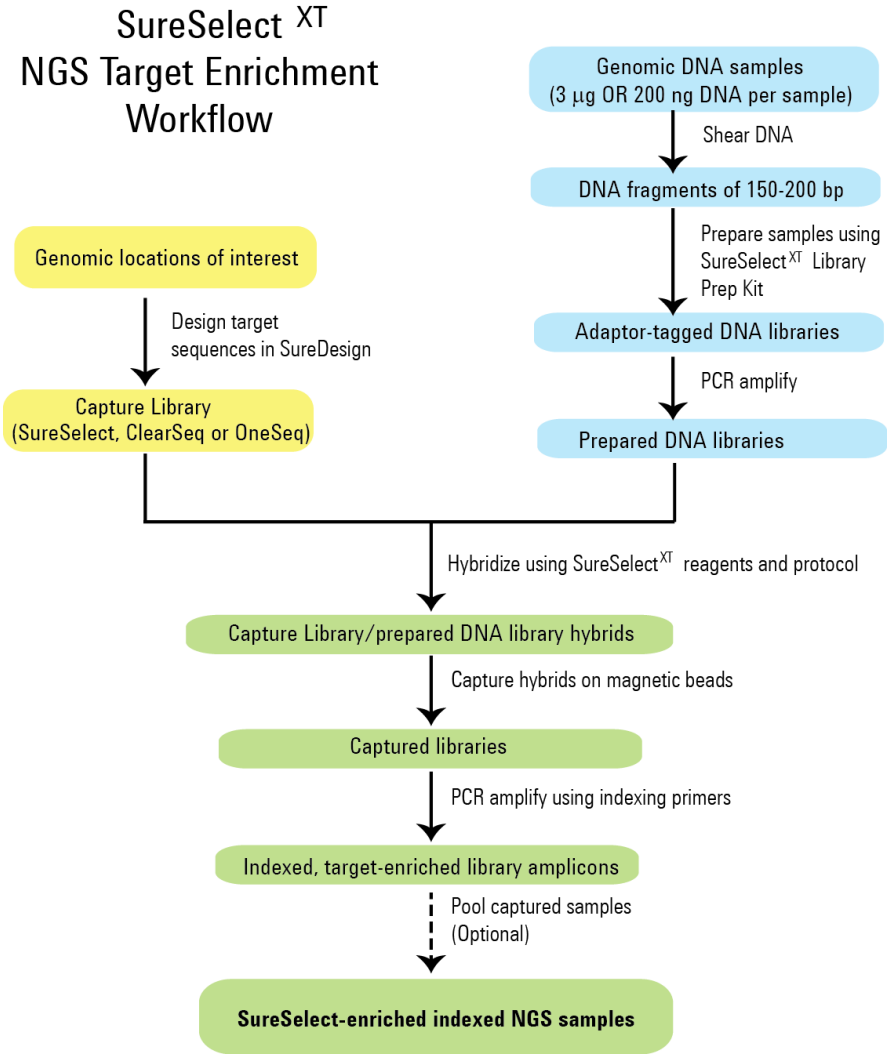


Figure 2 Overall sequencing sample preparation workflow.

Table 8 Overview of VWorks protocols and runsets used for 3 µg gDNA samples

Workflow Step (Protocol Chapter)	Substep	VWorks Protocols Used for Agilent NGS Workstation automation
Sample Preparation	Purify DNA using AMPure XP beads	AMPureXP_XT_ILM_v1.5.1.pro:Shearing-3 µg only
	Prepare adaptor-ligated DNA	LibraryPrep_XT_ILM_v1.5.1.rst
	Amplify adaptor-ligated DNA	Pre-CapturePCR_XT_ILM_3µg_v1.5.1.pro
	Purify DNA using AMPure XP beads	AMPureXP_XT_ILM_v1.5.1.pro:Pre-Capture PCR
Hybridization	Aliquot 750-ng of prepped libraries for hybridization	Aliquot_Libraries_v1.5.1.pro
	Hybridize prepped DNA to probe	Hybridization_v1.5.1.pro
	Capture and wash DNA hybrids	SureSelectCapture&Wash_v1.5.1.rst
Indexing	Add index tags by PCR	Post-CaptureIndexing_XT_ILM_v1.5.1.pro
	Purify DNA using AMPure XP beads	AMPureXP_XT_ILM_v1.5.1.pro:Post-Capture PCR

Table 9 Overview of VWorks protocols and runsets used for 200 ng gDNA samples

Workflow Step (Protocol Chapter)	Substep	VWorks Protocols Used for Agilent NGS Workstation automation
Sample Preparation	Prepare adaptor-ligated DNA	LibraryPrep_XT_ILM_v1.5.1.rst
	Amplify adaptor-ligated DNA	Pre-CapturePCR_XT_ILM_200ng_v1.5.1.pro
	Purify DNA using AMPure XP beads	AMPureXP_XT_ILM_v1.5.1.pro:Pre-Capture PCR
Hybridization	Aliquot 750-ng of prepped libraries for hybridization	Aliquot_Libraries_v1.5.1.pro
	Hybridize prepped DNA to probe	Hybridization_v1.5.1.pro
	Capture and wash DNA hybrids	SureSelectCapture&Wash_v1.5.1.rst
Indexing	Add index tags by PCR	Post-CaptureIndexing_XT_ILM_v1.5.1.pro
	Purify DNA using AMPure XP beads	AMPureXP_XT_ILM_v1.5.1.pro:Post-Capture PCR

Experimental Setup Considerations for Automated Runs

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

Table 10 Columns to Samples Equivalency

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

The number of columns or samples that may be processed using the supplied reagents (see Table 1) will depend on the experimental design. For greatest efficiency of reagent use, plan experiments using at least 3 columns per run. Each 96-reaction kit contains sufficient reagents for 96 reactions configured as 4 runs of 3 columns of samples per run.

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

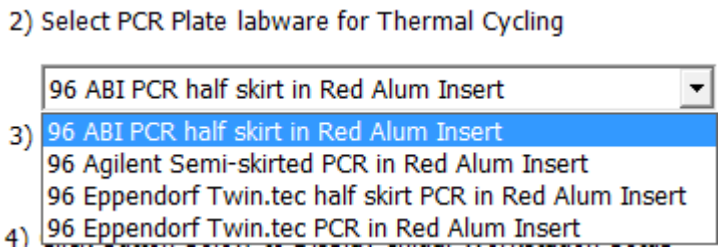
- The Agilent NGS Workstation processes samples column-wise beginning at column 1. gDNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- At the hybridization step (see Figure 2), you can add a different Probe Capture Library to each row of the plate. Plan your experiment such that each prepared DNA library corresponds to the appropriate probe row in the sample plate.
- For sample indexing after hybridization to the SureSelect library (see Figure 2), you will need to prepare a separate plate containing the indexing primers. Assign the wells to be indexed with their respective indexing primers during experimental design.
- For post-capture amplification (see Figure 2), different probes can require different amplification cycle numbers, based on the probe design sizes. It is most efficient to process similar-sized probes on the same plate. See Table 74 on page 135 to determine which probes may be amplified on the same plate.

Considerations for Equipment Setup

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

PCR Plate Type Considerations

Automation protocols include several liquid-handling steps in which reagents are dispensed to PCR plates in preparation for transfer to a thermal cycler. For these steps you must specify the PCR plate type to be used on the SureSelectXT_ILM_v1.5.1.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 11.



CAUTION

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

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

Table 11 Ordering information for supported PCR plates

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

3 Sample Preparation (3 µg DNA Samples)

Step 1. Shear DNA 38
Step 2. Purify sheared DNA using AMPure XP beads 40
Step 3. Assess sample quality (optional) 45
Step 4. Modify DNA ends for target enrichment 47
Step 5. Amplify adaptor-ligated libraries 55
Step 6. Purify amplified DNA using AMPure XP beads 63
Step 7. Assess Library DNA quantity and quality 66

This section contains instructions for the preparation of gDNA libraries from samples containing 3 µg of DNA. A separate protocol is provided on page 69 for 200 ng DNA samples.

This section contains instructions for gDNA library preparation specific to the Illumina paired-read sequencing platform and to automated processing using the Agilent NGS Workstation. For each sample to be sequenced, individual library preparations, hybridizations, and captures are performed in separate wells of a 96-well plate. The samples are then tagged by PCR with an index sequence. Depending on the target size of the SureSelect capture, multiple samples can be pooled and sequenced in a single lane using the Illumina-specified index tags that are provided with SureSelect^{XT} target enrichment kits.

Step 1. Shear DNA

For each DNA sample to be sequenced, prepare 1 library.

1. Use the Qubit dsDNA BR Assay to determine the concentration of your gDNA sample. Make sure the gDNA is of high quality (non-degraded, A_{260}/A_{280} is 1.8 to 2.0).

   Follow the instructions for the instrument.

2. Dilute 3 µg of high-quality gDNA with 1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) in a 1.5-mL LoBind tube to a total volume of 130 µL.

3. Set up the Covaris E-Series or S-Series 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 for at least 30 minutes, or according to the manufacturer's recommendations.

   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.

4. Put a Covaris microTUBE into the loading and unloading station.

   Keep the cap on the tube.

NOTE This protocol has been optimized using a Covaris model E220 instrument and 130-µl Covaris microTUBE or 96 microTUBE plate for shearing 130-µl DNA samples to a target DNA fragment size of 150 to 200 bp. To shear using a different Covaris instrument model/sample holder, or if your NGS workflow requires a different DNA fragment size, consult the manufacturer's literature for recommended shearing conditions.

5. Use a tapered pipette tip to slowly transfer the 130-µL DNA sample through the pre-split septum.

   Be careful not to introduce a bubble into the bottom of the tube.

6 Secure the microTUBE in the tube holder and shear the DNA with the settings in Table 12.

The target peak size is 150 to 200 bp.

Table 12 Shear settings for Covaris instruments (SonoLab software v7 or later)

Setting	Value
Duty Factor	10%
Peak Incident Power (PIP)	175
Cycles per Burst	200
Treatment Time	360 seconds
Bath Temperature	4° to 8° C

7 Put the Covaris microTUBE back into the loading and unloading station.

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

9 Transfer the sheared DNA into the wells of a 96-well Eppendorf plate, column-wise for processing on the Agilent NGS Workstation, in well order A1 to H1, then A2 to H2, ending with A12 to H12.

NOTE

SureSelect Automated Library Prep and Capture System runs may include 1, 2, 3, 4, 6, or 12 columns of the plate. See Using the Agilent NGS Workstation for SureSelect Target Enrichment for additional sample placement considerations.

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

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

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

Step 2. Purify sheared DNA using AMPure XP beads

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

Prepare the workstation and reagents

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
5. Mix the 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 185 µL of homogeneous AMPure XP beads per well, for each well to be processed.
7. Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water.
8. Prepare a separate Thermo Scientific reservoir containing 45 mL of freshly-prepared 70% ethanol.

9 Load the Labware MiniHub according to Table 13, using the plate orientations shown in Figure 3.

Table 13 Initial MiniHub configuration for AMPureXP_XT_ILM_v1.5.1.pro:Shearing-3 µg only

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	Empty	Empty	Empty
Shelf 4	Empty	Empty	Empty	Empty
Shelf 3	Empty	Empty Eppendorf Plate	Empty	Empty
Shelf 2	Empty	Nuclease-free water reservoir from step 7	AMPure XP beads in Nunc DeepWell plate from step 6	Empty
Shelf 1 (Bottom)	Empty	70% ethanol reservoir from step 8	Empty	Empty Tip Box

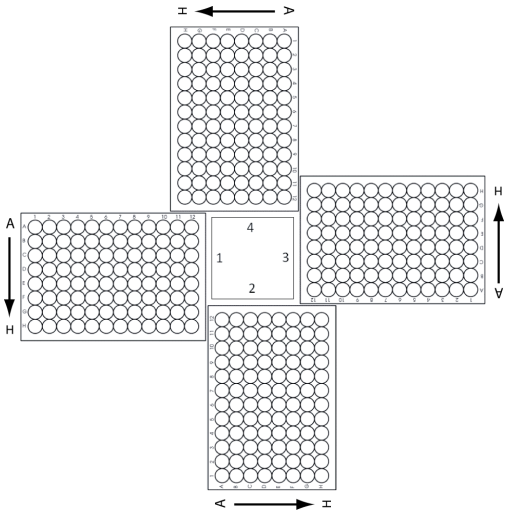


Figure 3 Agilent Labware MiniHub plate orientation. For Thermo Scientific reservoirs, place the notched corner facing the center of the hub.

10 Load the Bravo deck according to Table 14.

Table 14 Initial Bravo deck configuration for AMPureXP_XT_ILM_v1.5.1.pro:Shearing-3 µg only

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

11 Load the BenchCel Microplate Handling Workstation according to Table 15.

Table 15 Initial BenchCel configuration for AMPureXP_XT_ILM_v1.5.1.pro:Shearing-3 µg only

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

Run VWorks protocol *AMPureXP_XT_ILM_v1.5.1.pro:Shearing-3 µg only*

12 Open the SureSelect setup form using the XT_ILM_v1.5.1.VWForm shortcut on your desktop.

13 Log in to the VWorks software.

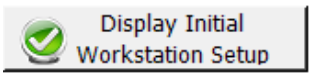
14 On the setup form, under **Select Protocol to Run**, select **AMPureXP_XT_ILM_v1.5.1.pro:Shearing-3 µg only.**

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

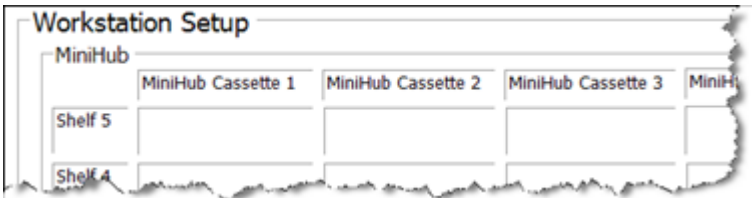
15 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate containing the sheared gDNA samples at position 9.

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

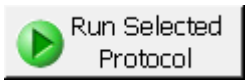
17 Click **Display Initial Workstation Setup**.



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



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



NOTE If workstation devices do not respond when you start the run, but activity is recorded in the Log, verify that VWorks is not running in Simulation mode. See page 29 for more information.

Running the AMPureXP purification protocol takes approximately 45 minutes. Once complete, the purified DNA samples are located in the Eppendorf plate at position 7 of the Bravo deck.

Step 3. Assess sample quality (optional)

Analysis of the purified sheared DNA samples prior to library preparation is optional. If you elect to include this step, follow the instructions below.

Option 1: Analysis using the 2100 Bioanalyzer and DNA 1000 Assay

Use a Bioanalyzer DNA 1000 chip and reagent kit and perform the assay according to the Agilent DNA 1000 Kit Guide.

1. Set up the 2100 Bioanalyzer as instructed in the reagent kit guide.
2. Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
3. Vortex the plate to mix samples in each well, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal.
4. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
5. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
6. Verify that the electropherogram shows the peak of DNA fragment size positioned between 150 to 200 bp. A sample electropherogram is shown in Figure 4.

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.

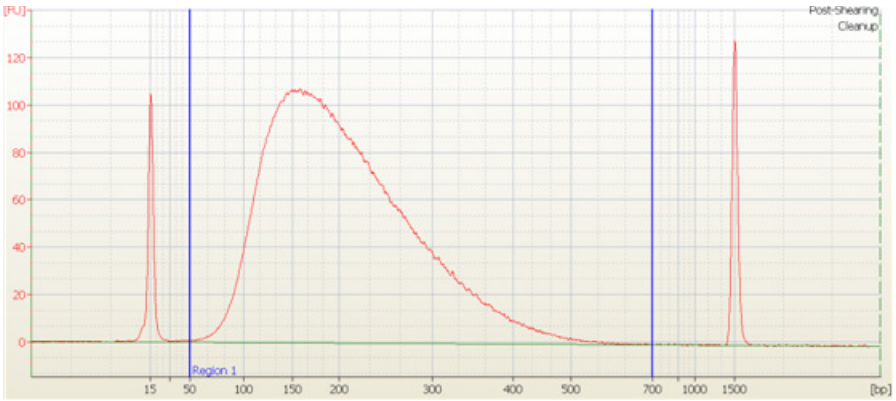


Figure 4 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and associated reagent kit. Perform the assay according to the Agilent D1000 Assay Quick Guide.

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

2 Vortex the plate to mix samples in each well, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal.

3 Prepare the TapeStation samples as instructed in the reagent kit guide. Use 1 µL of each sheared DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION For accurate quantitation, make sure to thoroughly mix the combined DNA and sample buffer by vortexing the assay plate or tube strip for 1 minute on the IKA MS3 vortex mixer provided with the 4200/4150 TapeStation system before loading the samples.

4 Load the sample plate or tube strips from step 3, the D1000 ScreenTape, and loading tips into the TapeStation as instructed in the reagent kit guide. Start the run.

5 Verify that the electropherogram shows the peak of DNA fragment size positioned between 150 to 200 bp. A sample electropherogram is shown in Figure 5.

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

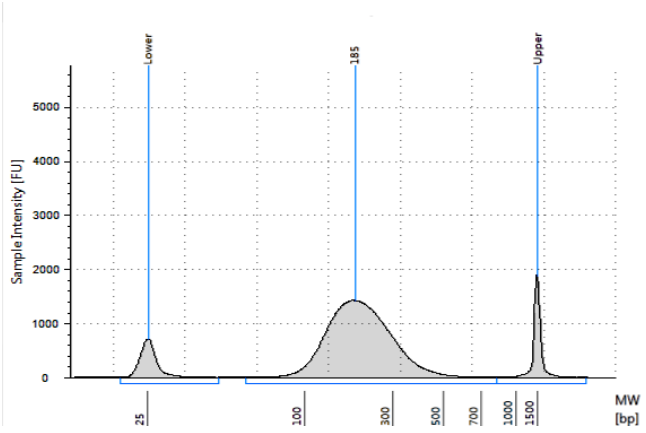


Figure 5 Analysis of sheared DNA using a D1000 ScreenTape.

Step 4. Modify DNA ends for target enrichment

In this step, the Agilent NGS Workstation completes the DNA end modification steps required for SureSelect target enrichment, including GA end-repair, A-tailing, and adaptor ligation. After the appropriate modification steps, the Agilent NGS Workstation purifies the prepared DNA using AMPure XP beads.

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 each table.

Prepare each master mix on ice.

Prepare the workstation

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

Prepare the SureSelect DNA end-repair master mix

4 Prepare the appropriate volume of end-repair master mix, according to Table 16. Mix well using a vortex mixer and keep on ice.

Table 16 Preparation of End-Repair Master Mix

SureSelect *XT* 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	35.2 µL	448.8 µL	748.0 µL	1047.2 µL	1346.4 µL	1944.8 µL	3889.6 µL
10X End-Repair Buffer	10.0 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1105.0 µL
dNTP mix	1.6 µL	20.4 µL	34.0 µL	47.6 µL	61.2 µL	88.4 µL	176.8 µL
T4 DNA Polymerase	1.0 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
Klenow DNA Polymerase	2.0 µL	25.5 µL	42.5 µL	59.5 µL	76.5 µL	110.5 µL	221.0 µL
T4 Polynucleotide Kinase	2.2 µL	28.1 µL	46.8 µL	65.5 µL	84.2 µL	121.6 µL	243.1 µL
Total Volume	**52 µL**	**663 µL**	**1105 µL**	**1547 µL**	**1989 µL**	**2873 µL**	**5746 µL**

Prepare the A-tailing master mix

5 Prepare the appropriate volume of A-tailing master mix, according to Table 17. Mix well using a vortex mixer and keep on ice.

Table 17 Preparation of A-Tailing Master Mix

SureSelect^{XT} 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	11.0 µL	187.0 µL	280.5 µL	374.0 µL	467.5 µL	654.5 µL	1262.3 µL
10x Klenow Polymerase Buffer	5.0 µL	85.0 µL	127.5 µL	170.0 µL	212.5 µL	297.5 µL	573.8 µL
dATP	1.0 µL	17.0 µL	25.5 µL	34.0 µL	42.5 µL	59.5 µL	114.8 µL
Exo (–) Klenow	3.0 µL	51.0 µL	76.5 µL	102.0 µL	127.5 µL	178.5 µL	344.3 µL
Total Volume	**20 µL**	**340 µL**	**510 µL**	**680 µL**	**850 µL**	**1190 µL**	**2295 µL**

Prepare the adaptor ligation master mix

6 Prepare the appropriate volume of adaptor ligation master mix, according to Table 18. Mix well using a vortex mixer and keep on ice.

Table 18 Preparation of Adaptor Ligation Master Mix **(use only for the 3 µg DNA input workflow)**

SureSelect^{XT} 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	15.5 µL	197.6 µL	329.4 µL	461.1 µL	592.9 µL	856.4 µL	1712.8 µL
5X T4 DNA Ligase Buffer	10.0 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1105.0 µL
SureSelect Adaptor Oligo Mix	10.0 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1105.0 µL
T4 DNA Ligase	1.5 µL	19.1 µL	31.9 µL	44.6 µL	57.4 µL	82.9 µL	165.8 µL
Total Volume	**37.0 µL**	**471.8 µL**	**786.3 µL**	**1100.8 µL**	**1415.3 µL**	**2044.3 µL**	**4088.5 µL**

Prepare the master mix source plate

7 In a Nunc DeepWell plate, prepare the master mix source plate containing the master mixes prepared in steps 3 to 5. Add the volumes indicated in Table 19 of each master mix 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 6.

Table 19 Preparation of the Master Mix Source Plate for LibraryPrep_XT_ILM_v1.5.1.rst

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 Master Mix	Column 1 (A1-H1)	76.4 µL	131.6 µL	186.9 µL	242.1 µL	352.6 µL	711.8 µL
A-Tailing Master Mix	Column 2 (A2-H2)	40.0 µL	61.3 µL	82.5 µL	103.8 µL	146.3µL	284.4 µL
Adaptor Ligation Master Mix	Column 3 (A3-H3)	54.3 µL	93.7 µL	133.0 µL	172.3 µL	250.9 µL	506.4 µL

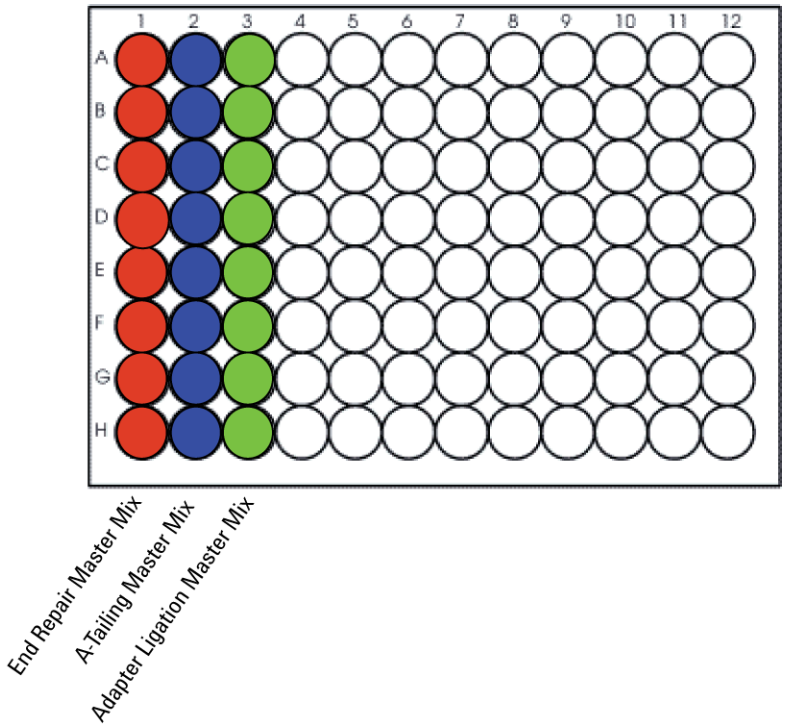


Figure 6 Configuration of the master mix source plate for LibraryPrep_XT_ILM_v1.5.1.rst

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.

Prepare the purification reagents

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

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

12 Prepare a separate Nunc DeepWell source plate for the beads by adding 370 µL of homogeneous AMPure XP beads per well, for each well to be processed.

13 Prepare a Thermo Scientific reservoir containing 20 mL of nuclease-free water.

14 Prepare a separate Thermo Scientific reservoir containing 150 mL of freshly-prepared 70% ethanol.

Load the Agilent NGS Workstation

15 Load the Labware MiniHub according to Table 20, using the plate orientations shown in Figure 3.

Table 20 Initial MiniHub configuration for LibraryPrep_XT_ILM_v1.5.1.rst

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	Empty Nunc DeepWell plate	Empty Nunc DeepWell plate	Empty
Shelf 4	Empty	Empty Eppendorf plate	Empty Eppendorf plate	Empty
Shelf 3	Empty	Empty	Empty	Empty Eppendorf plate
Shelf 2	Empty tip box	Nuclease-free water reservoir from step 13	AMPure XP beads in Nunc DeepWell plate from step 12	Empty
Shelf 1 (Bottom)	New tip box	70% ethanol reservoir from step 14	Empty	Empty tip box

16 Load the Bravo deck according to Table 21.

Table 21 Initial Bravo deck configuration for LibraryPrep_XT_ILM_v1.5.1.rst

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
6	Empty Eppendorf plate
7	Eppendorf plate containing purified gDNA samples
9	DNA End Modification Master Mix Source Plate, unsealed and seated on silver Nunc DeepWell insert

17 Load the BenchCel Microplate Handling Workstation according to Table 22.

Table 22 Initial BenchCel configuration for LibraryPrep_XT_ILM_v1.5.1.rst

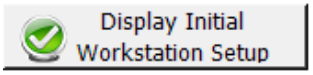
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	2 Tip boxes	Empty	Empty	Empty
2	4 Tip boxes	Empty	Empty	Empty
3	5 Tip boxes	Empty	Empty	Empty
4	7 Tip boxes	Empty	Empty	Empty
6	10 Tip boxes	Empty	Empty	Empty
12	11 Tip boxes	8 Tip boxes	Empty	Empty

Run VWorks runset LibraryPrep_XT_ILM_v1.5.1.rst

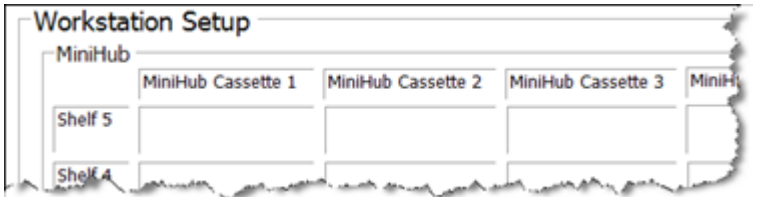
18 On the SureSelect setup form, under **Select Protocol to Run**, select **LibraryPrep_XT_ILM_v1.5.1.rst.**

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

20 Click **Display Initial Workstation Setup**.

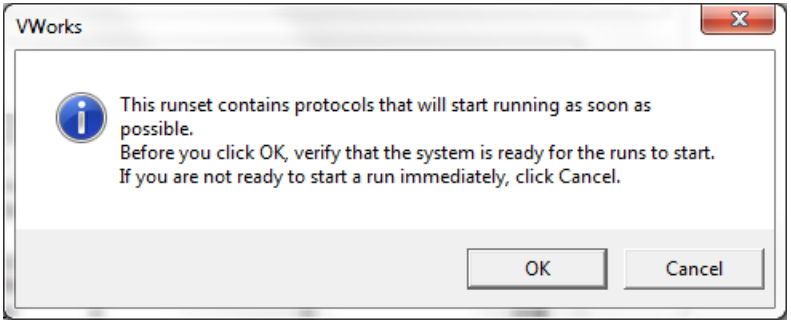


21 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

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



Running the LibraryPrep_XT_ILM_v1.5.1.rst runset takes approximately 3.5 hours. Once complete, the purified, adaptor-ligated DNA samples are located in the Eppendorf plate at position 7 of the Bravo deck.

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

Step 5. Amplify adaptor-ligated libraries

In this step, the Agilent NGS Workstation completes the liquid handling steps for amplification of the adaptor-ligated DNA samples. Afterward, you transfer the PCR plate to a thermal cycler for amplification.

In this protocol, one half of the adaptor-ligated DNA sample is removed from the Eppendorf sample plate for amplification. The remainder can be saved at 4°C for future use or amplification troubleshooting, if needed. Store the samples at –20°C for long-term storage.

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

Prepare the workstation

1. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
2. Leave tip boxes on shelves 1 and 2 in cassette 1 of the Labware MiniHub from the previous LibraryPrep_XT_ILM_v1.5.1.rst run. Otherwise, clear the remaining positions of the MiniHub and BenchCel of plates and tip boxes.
3. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.

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

4 Prepare the appropriate volume of pre-capture PCR Master Mix, according to Table 23. Mix well using a vortex mixer and keep on ice.

Table 23 Preparation of Pre-Capture PCR Master Mix **(use only for the 3 µg DNA input workflow)**

SureSelect^{XT} 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	21.0 µL	267.8 µL	446.3 µL	624.8 µL	803.3 µL	1160.3 µL	2320.5 µL
Herculase II 5X Reaction Buffer*	10.0 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1105 µL
dNTP mix*	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	27.6 µL	55.3 µL
SureSelect Primer† (Forward)	1.25 µL	15.9 µL	26.6 µL	37.2 µL	47.8 µL	69.1 µL	138.1 µL
SureSelect Indexing Pre-Capture PCR (Reverse) Primer‡	1.25 µL	15.9 µL	26.6 µL	37.2 µL	47.8 µL	69.1 µL	138.1 µL
Herculase II Polymerase	1.0 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
Total Volume	**35 µL**	**446.3 µL**	**743.8 µL**	**1041.3 µL**	**1338.8 µL**	**1933.8 µL**	**3867.5 µL**

* Included with the Herculase II Fusion DNA Polymerase. *Do not use the buffer or dNTP mix from any other kit.*

† Included in SureSelect XT Library Prep Kit ILM.

‡ Included in SureSelect XT Automation ILM Module Box 2. Ensure that the correct primer is selected from Box 2 at this step (do not use the SureSelect Indexing Post-Capture PCR (Forward) Primer).

5 Using the same Nunc DeepWell master mix source plate that was used for the LibraryPrep_XT_ILM_v1.5.1.rst run, add the volume of PCR Master Mix indicated in Table 24 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 7.

Table 24 Preparation of the Master Mix Source Plate for Pre-CapturePCR_XT_ILM_3µg_v1.5.1.pro

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)	51.4 µL	88.6 µL	125.8 µL	163.0 µL	237.3 µL	479.1 µL

NOTE If you are using a new DeepWell plate for the pre-capture PCR source plate (for example, when amplifying the second half of the adaptor-ligated DNA sample), leave columns 1 to 3 empty and add the PCR Master Mix to column 4 of the new plate.

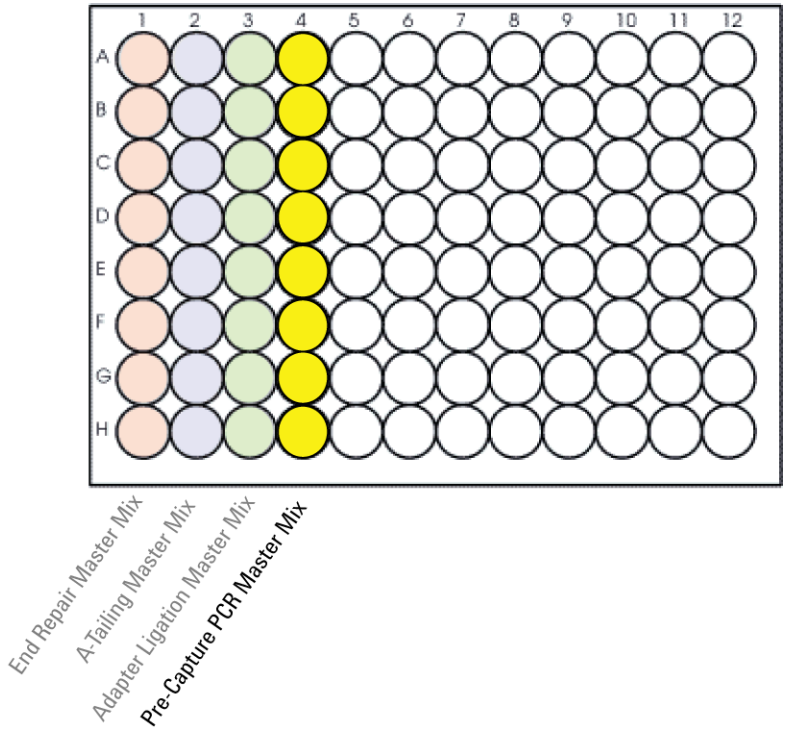


Figure 7 Configuration of the master mix source plate for Pre-CapturePCR_X-T_ILM_3µg_v1.5.1.pro. Columns 1-3 were used to dispense master mixes during the previous protocol.

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

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

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

Load the Agilent NGS Workstation

8 Load the Labware MiniHub according to Table 25, using the plate orientations shown in Figure 3.

Table 25 Initial MiniHub configuration for Pre-CapturePCR_XT_ILM_3µg_v1.5.1.pro

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty	Empty	Empty	Empty
Shelf 4	Empty	Empty	Empty	Empty
Shelf 3	Empty	Empty	Empty	Empty
Shelf 2	Waste tip box*	Empty	Empty	Empty
Shelf 1 (Bottom)	Clean tip box*	Empty	Empty	Empty tip box

* The waste tip box (Cassette 1, Shelf 2) and clean tip box (Cassette 1, Shelf 1) are retained from the LibraryPrep_XT_ILM_v1.5.1.rst run and reused here.

NOTE If you are using a new box of tips on shelf 1 of cassette 1 (for example, when amplifying the second half of the adaptor-ligated DNA sample), first remove the tips from columns 1 to 3 of the tip box. Any tips present in columns 1 to 3 of the tip box may be inappropriately loaded onto the Bravo platform pipette heads and may interfere with automated processing steps.

9 Load the Bravo deck according to Table 26.

Table 26 Initial Bravo deck configuration for Pre-CapturePCR_XT_ILM_3µg_v1.5.1.pro

Location	Content
6	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
7	Adaptor-ligated DNA samples in Eppendorf plate
9	Master mix plate containing PCR Master Mix in Column 4 (unsealed)

10 Load the BenchCel Microplate Handling Workstation according to Table 27.

Table 27 Initial BenchCel configuration for Pre-CapturePCR_XT_ILM_3µg_v1.5.1.pro

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

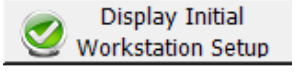
Run VWorks protocol Pre-CapturePCR_XT_ILM_3µg_v1.5.1.pro

11 On the SureSelect setup form, under **Select Protocol to Run**, select **Pre- CapturePCR_XT_ILM_3µg_v1.5.1.pro.**

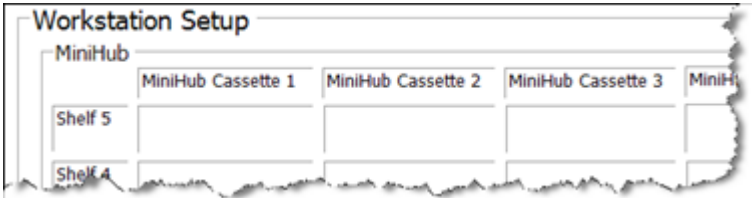
12 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

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 Workstation Setup**.



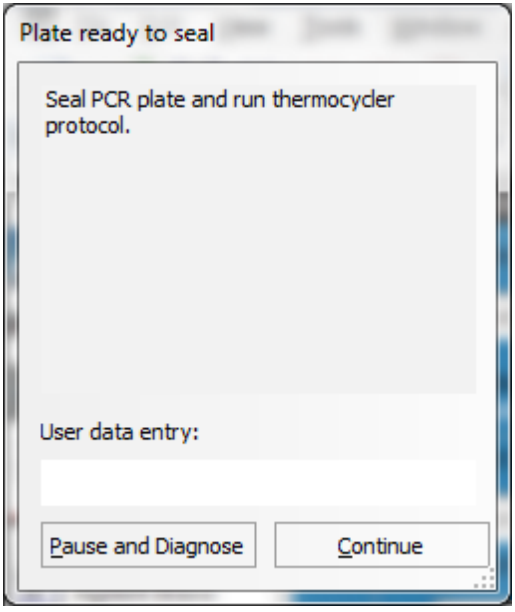
15 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

Running the Pre-CapturePCR_XT_ILM_3µg_v1.5.1.pro 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. The Eppendorf plate containing the remaining prepped DNA samples, which may be stored for future use at 4°C overnight, or at –20°C for long-term storage, is located at position 7 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 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.

19 Transfer the PCR plate to a thermal cycler and run the PCR amplification program shown in Table 28.

Table 28 Pre-Capture PCR cycling program **(use only for the 3 µg DNA input workflow)**

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	4 to 6	98°C	30 seconds
		65°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

NOTE

Different library preparations can produce slightly different results, based on varying DNA quality. In most cases, 5 cycles will produce an adequate yield for subsequent capture without introducing bias or non-specific products. If yield is too low or non-specific high molecular weight products are observed, adjust the number of cycles accordingly with the remaining library template.

Step 6. Purify amplified DNA using AMPure XP beads

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

Prepare the workstation and reagents

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

2 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.*

3 Mix the 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 95 µL of homogeneous AMPure XP beads per well, for each well to be processed.

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

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

7 Load the Labware MiniHub according to Table 29, using the plate orientations shown in Figure 3.

Table 29 Initial MiniHub configuration for AMPureXP_XT_ILM_v1.5.1.pro:Pre-Capture PCR

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	Empty	Empty	Empty
Shelf 4	Empty	Empty	Empty	Empty
Shelf 3	Empty	Empty Eppendorf Plate	Empty	Empty
Shelf 2	Empty	Nuclease-free water reservoir from step 5	AMPure XP beads in Nunc DeepWell plate from step 4	Empty
Shelf 1 (Bottom)	Empty	70% ethanol reservoir from step 6	Empty	Empty tip box

8 Load the Bravo deck according to Table 30.

Table 30 Initial Bravo deck configuration for AMPureXP_XT_ILM_v1.5.1.pro:Pre-Capture PCR

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

9 Load the BenchCel Microplate Handling Workstation according to Table 31.

Table 31 Initial BenchCel configuration for AMPureXP_XT_ILM_v1.5.1.pro:Pre-Capture PCR

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

Run VWorks protocol *AMPureXP_XT_ILM_v1.5.1.pro:Pre-Capture PCR*

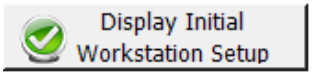
10 On the SureSelect setup form, under **Select Protocol to Run**, select **AMPureXP_XT_ILM_v1.5.1.pro:Pre- Capture PCR**.

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

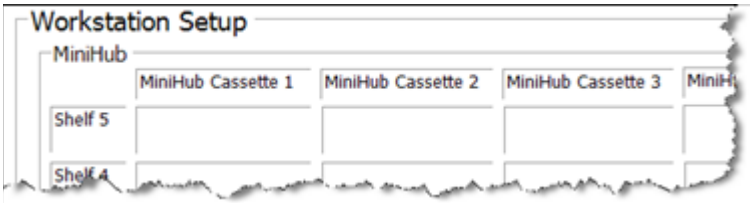
11 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate containing the amplified libraries at position 9.

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

13 Click **Display Initial Workstation Setup**.



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



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

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

Step 7. Assess Library DNA quantity and quality

The hybridization protocol in the following section requires 750 ng of each amplified DNA library. Measure the concentration of each library using one of the methods detailed below. Once DNA concentration for each sample is determined, calculate the volume of the library to be used for hybridization using the following formula:

Volume (µL) = 750 ng/concentration (ng/µL)

Option 1: Analysis using the Agilent 2100 Bioanalyzer and DNA 1000 Assay

Use a Bioanalyzer DNA 1000 chip and reagent kit and perform the assay according to the Agilent DNA 1000 Kit Guide.

1. Set up the 2100 Bioanalyzer as instructed in the reagent kit guide.
2. Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
3. Vortex the plate to mix samples in each well, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal.
4. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
5. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
6. Verify that the electropherogram shows the peak of DNA fragment size positioned between 225 to 275 bp. A sample electropherogram is shown in Figure 8.
7. Determine the concentration of the library (ng/µL) by integrating under the peak.

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.

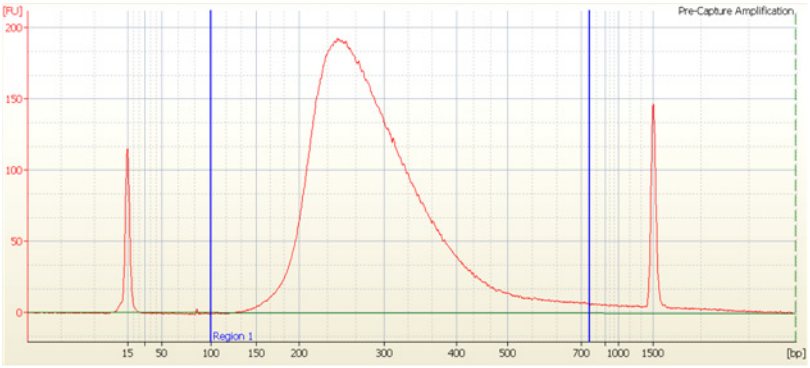


Figure 8 Analysis of amplified library DNA using a DNA 1000 assay.

Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and associated reagent kit. Perform the assay according to the Agilent D1000 Assay Quick Guide.

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

2 Vortex the plate to mix samples in each well, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal.

3 Prepare the TapeStation samples as instructed in the reagent kit guide. Use 1 µL of each amplified library DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION For accurate quantitation, make sure to thoroughly mix the combined DNA and sample buffer by vortexing the assay plate or tube strip for 1 minute on the IKA MS3 vortex mixer provided with the 4200/4150 TapeStation system before loading the samples.

4 Load the sample plate or tube strips from step 3, the D1000 ScreenTape, and loading tips into the TapeStation as instructed in the reagent kit guide. Start the run.

5 Verify that the electropherogram shows the peak of DNA fragment size positioned between 225 to 275 bp. A sample electropherogram is shown in Figure 9.

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

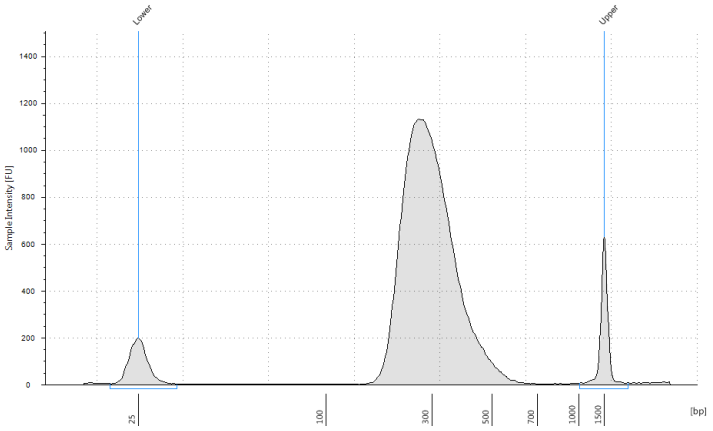


Figure 9 Analysis of amplified library DNA using a D1000 ScreenTape.

4 Sample Preparation (200 ng DNA Samples)

Step 1. Shear DNA 70
Step 2. Assess sample quality (optional) 73
Step 3. Modify DNA ends for target enrichment 75
Step 4. Amplify adaptor-ligated libraries 84
Step 5. Purify amplified DNA using AMPure XP beads 92
Step 6. Assess Library DNA quantity and quality 95

This section contains instructions for the preparation of gDNA libraries from samples containing 200 ng of DNA. A separate protocol is provided on page 37 for 3 µg DNA samples.

This section contains instructions for gDNA library preparation specific to the Illumina paired-read sequencing platform and to automated processing using the Agilent NGS Workstation. For each sample to be sequenced, individual library preparations, hybridizations, and captures are performed in separate wells of a 96-well plate. The samples are then tagged by PCR with an index sequence. Depending on the target size of the SureSelect capture, multiple samples can be pooled and sequenced in a single lane using the Illumina-specified index tags that are provided with SureSelect^{XT} target enrichment kits.

Step 1. Shear DNA

For each DNA sample to be sequenced, prepare 1 library.

1 Use the Qubit dsDNA BR Assay to determine the concentration of your gDNA sample. Make sure the gDNA is of high quality (non-degraded, A_{260}/A_{280} is 1.8 to 2.0).

   Follow the instructions for the instrument.

2 Dilute 200 ng of high-quality gDNA with 1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) in a 1.5-mL LoBind tube to a total volume of 50 µL.

3 Set up the Covaris E-Series or S-Series 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 for at least 30 minutes, or according to the manufacturer's recommendations.

   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.

4 Put a Covaris microTUBE into the loading and unloading station.

   Keep the cap on the tube.

NOTE This protocol has been optimized using a Covaris model E220 instrument and 130-µl Covaris microTUBE or 96 microTUBE plate for shearing 50-µl DNA samples to a target DNA fragment size of 150 to 200 bp. To shear using a different Covaris instrument model/sample holder (e.g. 50-µl microTUBE), or if your NGS workflow requires a different DNA fragment size, consult the manufacturer's literature for recommended shearing conditions.

5 Use a tapered pipette tip to slowly transfer the 50-µL DNA sample through the pre-split septum.

   Be careful not to introduce a bubble into the bottom of the tube.

6 Secure the microTUBE in the tube holder and shear the DNA with the settings in Table 32.

The target peak size is 150 to 200 bp.

Table 32 Shear settings for Covaris instruments (SonoLab software v7 or later)

Setting	Value
Duty Factor	10%
Peak Incident Power (PIP)	175
Cycles per Burst	200
Treatment Time	360 seconds*
Bath Temperature	4° to 8° C

* For more complete shearing when using individual Covaris microTUBEs, the 360-second treatment time may be completed in two rounds of 180 seconds each. After completing the first round of shearing for 180 seconds, spin the microTUBE briefly to collect the liquid, then shear the DNA for an additional 180 seconds.

7 Put the Covaris microTUBE back into the loading and unloading station.

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

9 Transfer the sheared DNA into the wells of a 96-well Eppendorf plate, column-wise for processing on the Agilent NGS Workstation, in well order A1 to H1, then A2 to H2, ending with A12 to H12.

NOTE

SureSelect Automated Library Prep and Capture System runs may include 1, 2, 3, 4, 6, or 12 columns of the plate. See Using the Agilent NGS Workstation for SureSelect Target Enrichment for additional sample placement considerations.

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

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

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

CAUTION The Sample Preparation protocol for 200 ng gDNA samples does not include the post-shear purification step that is included in the Sample Preparation protocol for 3 µg gDNA samples.

If you wish to analyze the sheared DNA fragment size prior to library preparation, use the optional protocol on page 73. Otherwise, proceed directly to "Step 3. Modify DNA ends for target enrichment" on page 75.

Step 2. Assess sample quality (optional)

Analysis of the sheared DNA samples prior to library preparation is optional. If you elect to include this step, follow the instructions below.

Option 1: Analysis using the 2100 Bioanalyzer and High Sensitivity DNA Assay

Use the Bioanalyzer High Sensitivity DNA Assay to analyze the 200-ng sheared DNA samples. Perform the assay according to the High Sensitivity DNA Kit Guide.

1. Set up the 2100 Bioanalyzer as instructed in the reagent kit guide.
2. Vortex the plate to mix samples in each well, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal.
3. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
4. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
5. Verify that the electropherogram shows the peak of DNA fragment size positioned between 120 to 150 bp. A sample electropherogram is shown in Figure 10.

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.

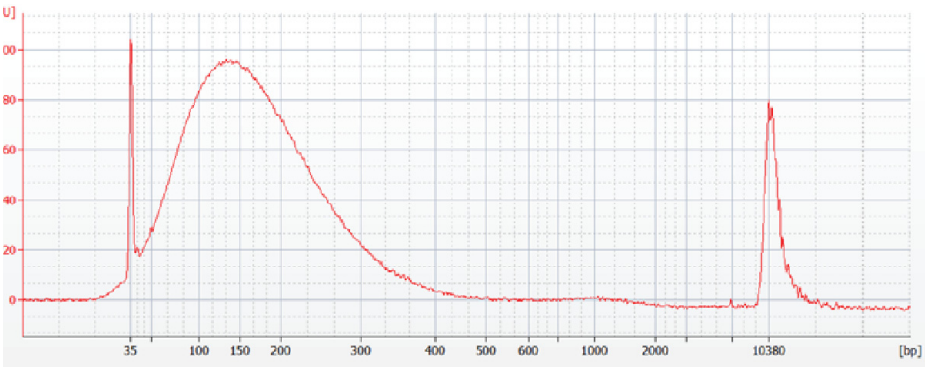


Figure 10 Analysis of sheared DNA using a High Sensitivity DNA Bioanalyzer assay.

Option 2: Analysis using an Agilent TapeStation and High Sensitivity D1000 ScreenTape

Use a High Sensitivity D1000 ScreenTape and associated reagent kit to analyze the 200-ng sheared DNA samples. Perform the assay according to the Agilent High Sensitivity D1000 Assay Quick Guide.

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

2 Vortex the plate to mix samples in each well, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal.

3 Prepare the TapeStation samples as instructed in the reagent kit guide. Use 2 µL of each sheared DNA sample diluted with 2 µL of High Sensitivity D1000 sample buffer for the analysis.

CAUTION For accurate quantitation, make sure to thoroughly mix the combined DNA and sample buffer by vortexing the assay plate or tube strip for 1 minute on the IKA MS3 vortex mixer provided with the 4200/4150 TapeStation system before loading the samples.

4 Load the sample plate or tube strips from step 3, the High Sensitivity D1000 ScreenTape, and loading tips into the TapeStation instrument as instructed in the reagent kit guide. Start the run.

5 Verify that the electropherogram shows the peak of DNA fragment size positioned between 120 to 150 bp. A sample electropherogram is shown in Figure 11.

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

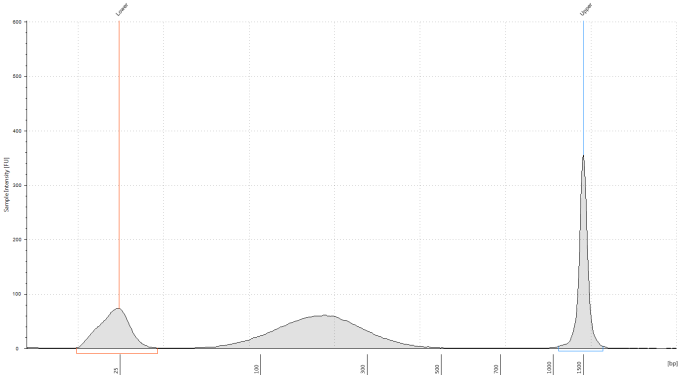


Figure 11 Analysis of sheared DNA using a High Sensitivity D1000 ScreenTape.

Step 3. Modify DNA ends for target enrichment

In this step, the Agilent NGS Workstation completes the DNA end modification steps required for SureSelect target enrichment, including GA end-repair, A-tailing, and adaptor ligation. After the appropriate modification steps, the Agilent NGS Workstation purifies the prepared DNA using AMPure XP beads.

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 each table.

Prepare each master mix on ice.

CAUTION

The Library Prep automation protocol for 200 ng gDNA samples differs from the 3 µg gDNA protocol in the amount of SureSelect Adaptor Oligo Mix used in the adaptor ligation master mix. Be sure to use the master mix preparation table provided on page 77 for 200 ng DNA samples.

Prepare the workstation

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

Prepare the SureSelect DNA end-repair master mix

4 Prepare the appropriate volume of end-repair master mix, according to Table 33. Mix well using a vortex mixer and keep on ice.

Table 33 Preparation of End-Repair Master Mix

SureSelect XT 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	35.2 µL	448.8 µL	748.0 µL	1047.2 µL	1346.4 µL	1944.8 µL	3889.6 µL
10X End-Repair Buffer	10.0 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1105.0 µL
dNTP mix	1.6 µL	20.4 µL	34.0 µL	47.6 µL	61.2 µL	88.4 µL	176.8 µL
T4 DNA polymerase	1.0 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
Klenow DNA polymerase	2.0 µL	25.5 µL	42.5 µL	59.5 µL	76.5 µL	110.5 µL	221.0 µL
T4 Polynucleotide Kinase	2.2 µL	28.1 µL	46.8 µL	65.5 µL	84.2 µL	121.6 µL	243.1 µL
Total Volume	**52 µL**	**663 µL**	**1105 µL**	**1547 µL**	**1989 µL**	**2873 µL**	**5746 µL**

Prepare the A-tailing master mix

5 Prepare the appropriate volume of A-tailing master mix, according to Table 34. Mix well using a vortex mixer and keep on ice.

Table 34 Preparation of A-Tailing Master Mix

SureSelect XT 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	11.0 µL	187.0 µL	280.5 µL	374.0 µL	467.5 µL	654.5 µL	1262.3 µL
10x Klenow Polymerase Buffer	5.0 µL	85.0 µL	127.5 µL	170.0 µL	212.5 µL	297.5 µL	573.8 µL
dATP	1.0 µL	17.0 µL	25.5 µL	34.0 µL	42.5 µL	59.5 µL	114.8 µL
Exo (–) Klenow	3.0 µL	51.0 µL	76.5 µL	102.0 µL	127.5 µL	178.5 µL	344.3 µL
Total Volume	**20 µL**	**340 µL**	**510 µL**	**680 µL**	**850 µL**	**1190 µL**	**2295 µL**

Prepare the adaptor ligation master mix

6 Prepare the appropriate volume of adaptor ligation master mix, according to Table 35. Mix well using a vortex mixer and keep on ice.

Table 35 Preparation of Adaptor Ligation Master Mix **(use only for the 200 ng DNA input workflow)**

SureSelect XT 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	24.5 µL	312.4 µL	520.6 µL	728.9 µL	937.1 µL	1353.6 µL	2707.3 µL
5X T4 DNA Ligase Buffer	10.0 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1105.0 µL
SureSelect Adaptor Oligo Mix*	1.0 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
T4 DNA Ligase	1.5 µL	19.1 µL	31.9 µL	44.6 µL	57.4 µL	82.9 µL	165.8 µL
Total Volume	**37.0 µL**	**471.8 µL**	**786.3 µL**	**1100.8 µL**	**1415.3 µL**	**2044.3 µL**	**4088.5 µL**

* Previously labeled as InPE Adaptor Oligo Mix.

Prepare the master mix source plate

7 In a Nunc DeepWell plate, prepare the master mix source plate containing the master mixes prepared in steps 3 to 5. Add the volumes indicated in Table 36 of each master mix 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 12.

Table 36 Preparation of the Master Mix Source Plate for LibraryPrep_XT_ILM_v1.5.1.rst

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 Master Mix	Column 1 (A1-H1)	76.4 µL	131.6 µL	186.9 µL	242.1 µL	352.6 µL	711.8 µL
A-Tailing Master Mix	Column 2 (A2-H2)	40.0 µL	61.3 µL	82.5 µL	103.8 µL	146.3µL	284.4 µL
Adaptor Ligation Master Mix	Column 3 (A3-H3)	54.3 µL	93.7 µL	133.0 µL	172.3 µL	250.9 µL	506.4 µL

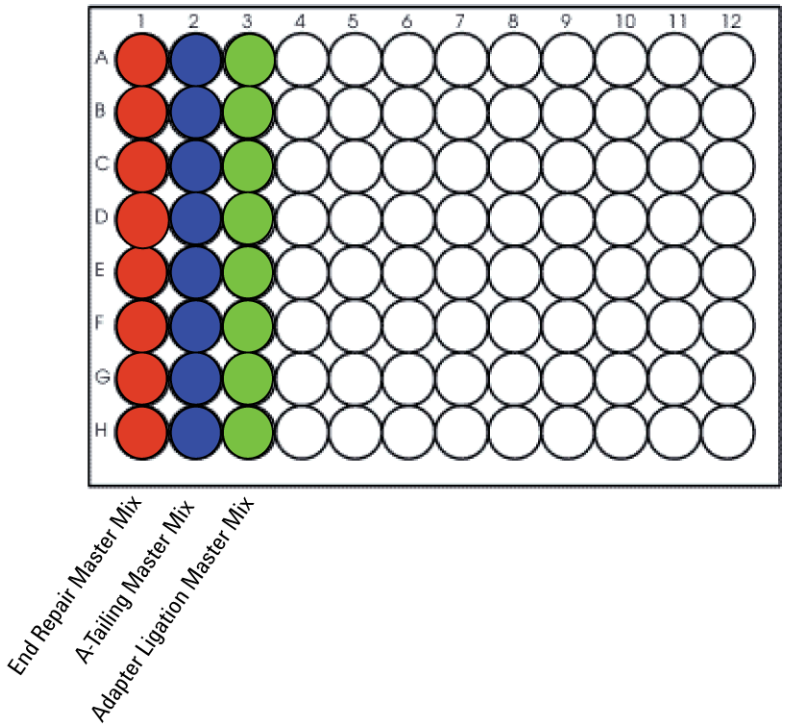


Figure 12 Configuration of the master mix source plate for LibraryPrep_XT_ILM_v1.5.1.rst

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.

Prepare the purification reagents

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

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

12 Prepare a separate Nunc DeepWell source plate for the beads by adding 370 µL of homogeneous AMPure XP beads per well, for each well to be processed.

13 Prepare a Thermo Scientific reservoir containing 20 mL of nuclease-free water.

14 Prepare a separate Thermo Scientific reservoir containing 150 mL of freshly-prepared 70% ethanol.

Load the Agilent NGS Workstation

15 Load the Labware MiniHub according to Table 37, using the plate orientations shown in Figure 13.

Table 37 Initial MiniHub configuration for LibraryPrep_XT_ILM_v1.5.1.rst

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	Empty Nunc DeepWell plate	Empty Nunc DeepWell plate	Empty
Shelf 4	Empty	Empty Eppendorf plate	Empty Eppendorf plate	Empty
Shelf 3	Empty	Empty	Empty	Empty Eppendorf plate
Shelf 2	Empty tip box	Nuclease-free water reservoir from step 13	AMPure XP beads in Nunc DeepWell plate from step 12	Empty
Shelf 1 (Bottom)	New tip box	70% ethanol reservoir from step 14	Empty	Empty tip box

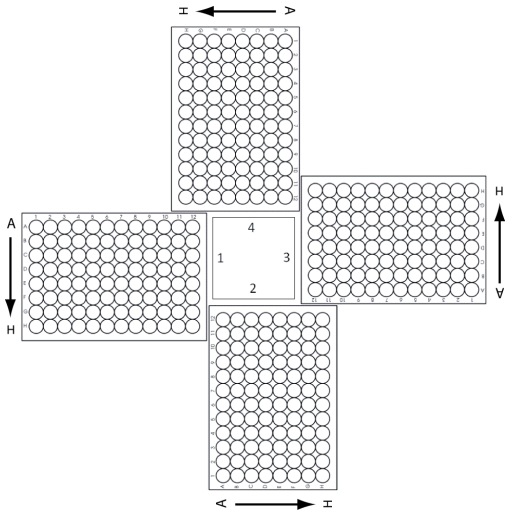


Figure 13 Agilent Labware MiniHub plate orientation. For Thermo Scientific reservoirs, place the notched corner facing the center of the hub.

16 Load the Bravo deck according to Table 38.

Table 38 Initial Bravo deck configuration for LibraryPrep_XT_ILM_v1.5.1.rst

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
6	Empty Eppendorf plate
7	Eppendorf plate containing sheared gDNA samples (unsealed)
9	DNA End Modification Master Mix Source Plate (unsealed) seated on silver Nunc DeepWell insert

17 Load the BenchCel Microplate Handling Workstation according to Table 39.

Table 39 Initial BenchCel configuration for LibraryPrep_XT_ILM_v1.5.1.rst

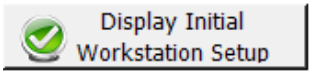
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	2 Tip boxes	Empty	Empty	Empty
2	4 Tip boxes	Empty	Empty	Empty
3	5 Tip boxes	Empty	Empty	Empty
4	7 Tip boxes	Empty	Empty	Empty
6	10 Tip boxes	Empty	Empty	Empty
12	11 Tip boxes	8 Tip boxes	Empty	Empty

Run VWorks runset LibraryPrep_XT_ILM_v1.5.1.rst

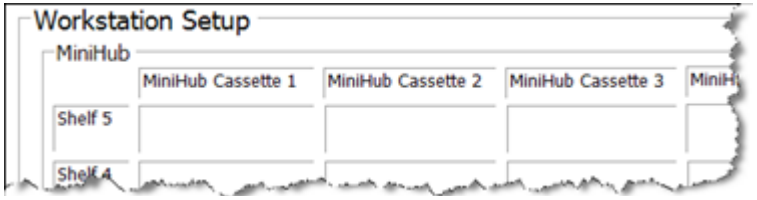
18 On the SureSelect setup form, under **Select Protocol to Run**, select **LibraryPrep_XT_ILM_v1.5.1.rst.**

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

20 Click **Display Initial Workstation Setup**.

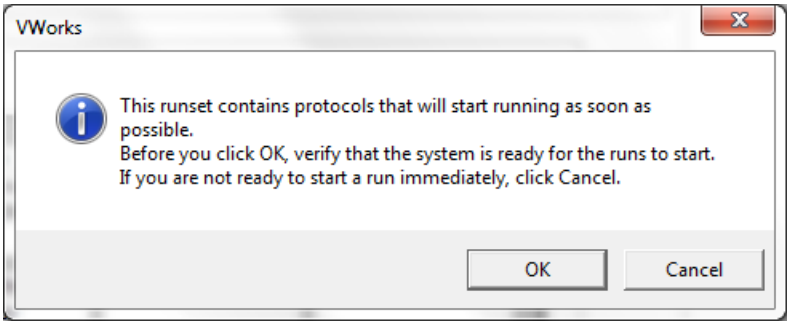


21 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

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



Running the LibraryPrep_XT_ILM_v1.5.1.rst runset takes approximately 3.5 hours. Once complete, the purified, adaptor-ligated DNA samples are located in the Eppendorf plate at position 7 of the Bravo deck.

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

Step 4. Amplify adaptor-ligated libraries

In this step, the Agilent NGS Workstation completes the liquid handling steps for amplification of the adaptor-ligated DNA samples. Afterward, you transfer the PCR plate to a thermal cycler for amplification.

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

Prepare the workstation

1. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
2. Leave tip boxes on shelves 1 and 2 in cassette 1 of the Labware MiniHub from the previous LibraryPrep_XT_ILM_v1.5.1.rst run. Otherwise, clear the remaining positions of the MiniHub and BenchCel of plates and tip boxes.
3. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.

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

4 Prepare the appropriate volume of pre-capture PCR Master Mix, according to Table 40. Mix well using a vortex mixer and keep on ice.

Table 40 Preparation of Pre-Capture PCR Master Mix **(use only for the 200 ng DNA input workflow)**

SureSelect^{XT} 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	6.0 µL	76.5 µL	127.5 µL	178.5 µL	229.5 µL	331.5 µL	663.0 µL
Herculase II 5X Reaction Buffer*	10.0 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1105 µL
dNTP mix*	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	27.6 µL	55.3 µL
SureSelect Primer† (Forward)	1.25 µL	15.9 µL	26.6 µL	37.2 µL	47.8 µL	69.1 µL	138.1 µL
SureSelect Indexing Pre-Capture PCR (Reverse) Primer‡	1.25 µL	15.9 µL	26.6 µL	37.2 µL	47.8 µL	69.1 µL	138.1 µL
Herculase II Polymerase	1.0 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
Total Volume	**20 µL**	**255 µL**	**425 µL**	**595 µL**	**765 µL**	**1105 µL**	**2210 µL**

* Included with the Herculase II Fusion DNA Polymerase. *Do not use the buffer or dNTP mix from any other kit.*

† Included in SureSelect XT Library Prep Kit ILM.

‡ Included in SureSelect XT Automation ILM Module Box 2. Ensure that the correct primer is selected from Box 2 at this step (do not use the SureSelect Indexing Post-Capture PCR (Forward) Primer).

5 Using the same Nunc DeepWell master mix source plate that was used for the LibraryPrep_XT_ILM_v1.5.1.rst run, add the volume of PCR Master Mix indicated in Table 41 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 14.

Table 41 Preparation of the Master Mix Source Plate for Pre-CapturePCR_XT_ILM_200ng_v1.5.1.pro

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)	29.4 µL	50.6 µL	71.9 µL	93.1 µL	135.6 µL	273.8 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.

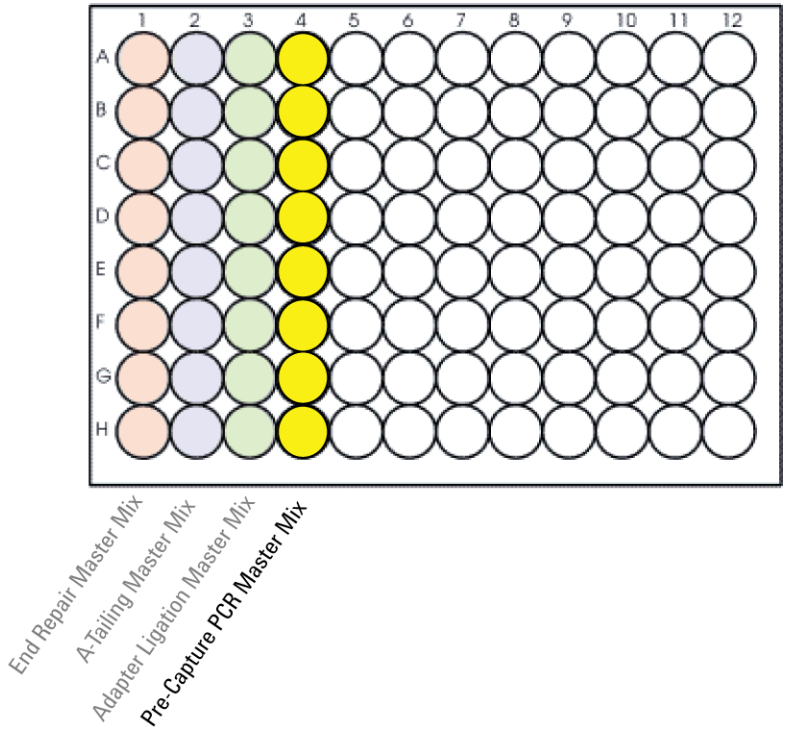


Figure 14 Configuration of the master mix source plate for Pre-CapturePCR_X-T_ILM_200ng_v1.5.1.pro. Columns 1-3 were used to dispense master mixes during the previous protocol.

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

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

NOTE

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

Load the Agilent NGS Workstation

8 Load the Labware MiniHub according to Table 42, using the plate orientations shown in Figure 13.

Table 42 Initial MiniHub configuration for Pre-CapturePCR_XT_ILM_200ng_v1.5.1.pro

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty	Empty	Empty	Empty
Shelf 4	Empty	Empty	Empty	Empty
Shelf 3	Empty	Empty	Empty	Empty
Shelf 2	Waste tip box*	Empty	Empty	Empty
Shelf 1 (Bottom)	Clean tip box*	Empty	Empty	Empty tip box

* The waste tip box (Cassette 1, Shelf 2) and clean tip box (Cassette 1, Shelf 1) are retained from the LibraryPrep_XT_ILM_v1.5.1.rst run and reused here.

NOTE If you are using a new box of tips on shelf 1 of cassette 1, first remove the tips from columns 1 to 3 of the tip box. Any tips present in columns 1 to 3 of the tip box may be inappropriately loaded onto the Bravo platform pipette heads and may interfere with automated processing steps.

9 Load the Bravo deck according to Table 43.

Table 43 Initial Bravo deck configuration for Pre-CapturePCR_XT_ILM_200ng_v1.5.1.pro

Location	Content
6	Empty PCR plate seated on red insert (PCR plate type must be specified on setup form under step 2)
7	Adaptor-ligated DNA samples in Eppendorf plate
9	Master mix plate containing PCR Master Mix in Column 4 (unsealed)

10 Load the BenchCel Microplate Handling Workstation according to Table 44.

Table 44 Initial BenchCel configuration for Pre-CapturePCR_XT_ILM_200ng_v1.5.1.pro

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

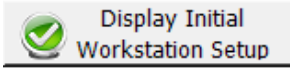
Run VWorks protocol Pre-CapturePCR_XT_ILM_200ng_v1.5.1.pro

11 On the SureSelect setup form, under **Select Protocol to Run**, select **Pre- CapturePCR_XT_ILM_200ng_v1.5.1.pro.**

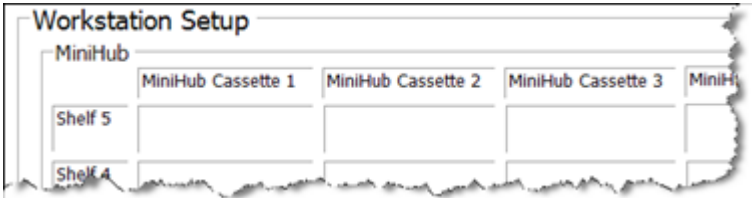
12 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

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

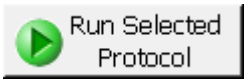
14 Click **Display Initial Workstation Setup**.



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

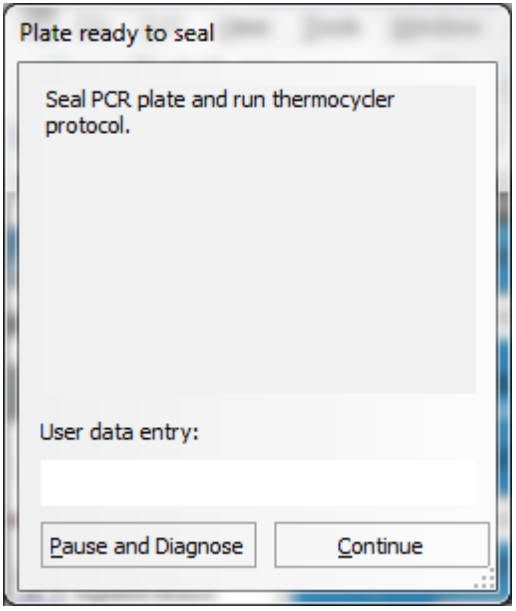


16 When verification is complete, click **Run Selected Protocol**.



Running the Pre-CapturePCR_XT_ILM_200ng_v1.5.1.pro 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.

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

Transfer the PCR plate to a thermal cycler and run the PCR amplification program shown in Table 45.

Table 45 Pre-Capture PCR cycling program **(use only for the 200 ng DNA input workflow)**

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10	98°C	30 seconds
		65°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

Step 5. Purify amplified DNA using AMPure XP beads

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

Prepare the workstation and reagents

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

2 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.*

3 Mix the 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 95 µL of homogeneous AMPure XP beads per well, for each well to be processed.

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

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

7 Load the Labware MiniHub according to Table 46, using the plate orientations shown in Figure 13.

Table 46 Initial MiniHub configuration for AMPureXP_XT_ILM_v1.5.1.pro:Pre-Capture PCR

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	Empty	Empty	Empty
Shelf 4	Empty	Empty	Empty	Empty
Shelf 3	Empty	Empty Eppendorf Plate	Empty	Empty
Shelf 2	Empty	Nuclease-free water reservoir from step 5	AMPure XP beads in Nunc DeepWell plate from step 4	Empty
Shelf 1 (Bottom)	Empty	70% ethanol reservoir from step 6	Empty	Empty tip box

8 Load the Bravo deck according to Table 47.

Table 47 Initial Bravo deck configuration for AMPureXP_XT_ILM_v1.5.1.pro:Pre-Capture PCR

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

9 Load the BenchCel Microplate Handling Workstation according to Table 48.

Table 48 Initial BenchCel configuration for AMPureXP_XT_ILM_v1.5.1.pro:Pre-Capture PCR

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

Run VWorks protocol *AMPureXP_XT_ILM_v1.5.1.pro:Pre-Capture PCR*

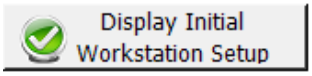
10 On the SureSelect setup form, under **Select Protocol to Run**, select **AMPureXP_XT_ILM_v1.5.1.pro:Pre- Capture PCR**.

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

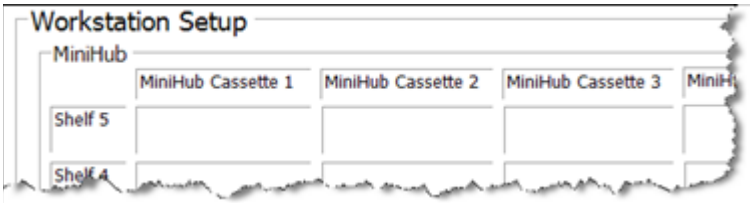
11 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate containing the amplified libraries at position 9.

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

13 Click **Display Initial Workstation Setup**.



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



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

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

Step 6. Assess Library DNA quantity and quality

The hybridization protocol in the following section requires 750 ng of each amplified DNA library. Measure the concentration of each library using one of the methods detailed below. Once DNA concentration for each sample is determined, calculate the volume of the library to be used for hybridization using the following formula:

Volume (µL) = 750 ng/concentration (ng/µL)

Option 1: Analysis using the Agilent 2100 Bioanalyzer and DNA 1000 Assay

Use a Bioanalyzer DNA 1000 chip and reagent kit to analyze the amplified libraries. Perform the assay according to the Agilent DNA 1000 Kit Guide.

1. Set up the 2100 Bioanalyzer as instructed in the reagent kit guide.
2. Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
3. Vortex the plate to mix samples in each well, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal.
4. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
5. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
6. Verify that the electropherogram shows the peak of DNA fragment size positioned between 225 to 275 bp. A sample electropherogram is shown in Figure 15.
7. Determine the concentration of the library (ng/µL) by integrating under the peak.

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.

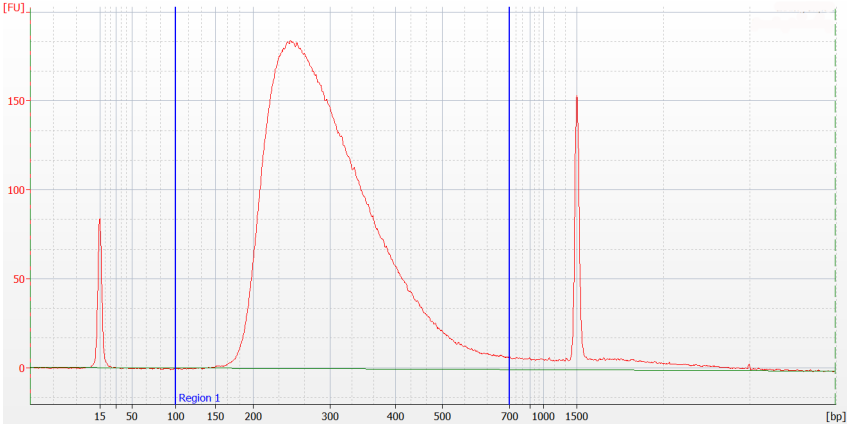


Figure 15 Analysis of amplified library DNA using a DNA 1000 assay.

Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and associated reagent kit to analyze the amplified libraries. Perform the assay according to the Agilent D1000 Assay Quick Guide.

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

2 Vortex the plate to mix samples in each well, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal.

3 Prepare the TapeStation samples as instructed in the reagent kit guide. Use 1 µL of each amplified library DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION

For accurate quantitation, make sure to thoroughly mix the combined DNA and sample buffer by vortexing the assay plate or tube strip for 1 minute on the IKA MS3 vortex mixer provided with the 4200/4150 TapeStation system before loading the samples.

4 Load the sample plate or tube strips from step 3, the D1000 ScreenTape, and loading tips into the TapeStation as instructed in the reagent kit guide. Start the run.

5 Verify that the electropherogram shows the peak of DNA fragment size positioned between 225 to 275 bp. A sample electropherogram is shown in Figure 16.

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

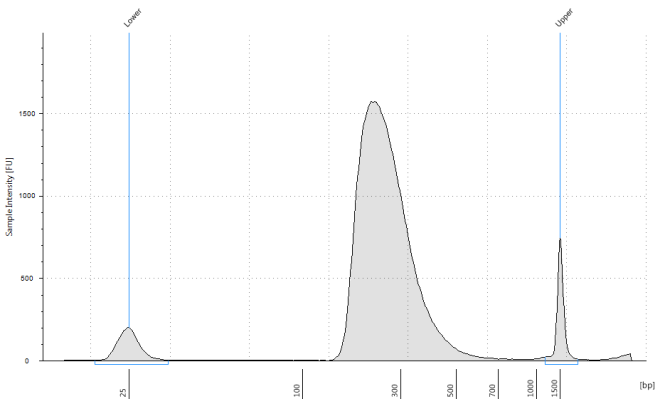


Figure 16 Analysis of amplified library DNA using a D1000 ScreenTape.

5 Hybridization

Step 1. Aliquot prepped DNA samples for hybridization 100
Step 2. Hybridize DNA samples to the probe 103
Step 3. Capture the hybridized DNA 117

This chapter describes the steps to combine the prepped library with the blocking agents and the Probe Capture Library. Each DNA library sample must be hybridized and captured individually prior to addition of the indexing tag by PCR.

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.

Each hybridization reaction will contain 750 ng of the prepped gDNA sample. Before starting the hybridization step, you must create a table containing instructions for the Agilent NGS Workstation indicating the volume of each sample required for a 750-ng aliquot.

1 Create a .csv (comma separated value) file with the headers shown in Figure 17. 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) equivalent to 750 ng DNA for each sample. These values are determined from the concentration values obtained from Bioanalyzer or TapeStation traces in the previous section. For all empty wells on the plate, enter the value 0, as shown in Figure 17; **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 17 Sample spreadsheet for 750-ng sample aliquot for 1-column run.

NOTE

You can find a sample spreadsheet in the directory **C: > VWorks Workspace > NGS Option B > XT Illumina_1.5.1> Aliquot Library Input Files > 750ng_transfer_full_plate_template_xlsx.**

The 750ng_transfer_full_plate_template.xlsx file may be copied and used as a template for creating the .csv files for each Aliquot_Libraries_v1.5.1.pro run. If you are using the sample file as a template for runs with fewer than 12 columns, be sure to retain rows for all 96 wells, and populate the Volume column with 0 for unused wells.

3 Load the .csv file onto the PC containing the VWorks software into a suitable folder, such as **C: > VWorks Workspace > NGS Option B > XT Illumina_1.5.1 > Aliquot Library Input Files.**

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

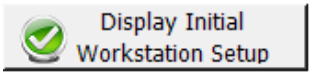
5 Load the Bravo deck according to Table 49.

Table 49 Initial Bravo deck configuration for Aliquot_Libraries_v1.5.1.pro

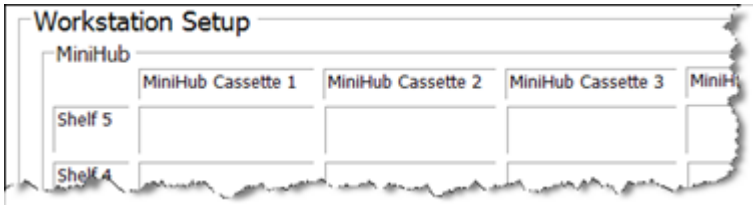
Location	Content
5	Empty Eppendorf plate
6	Empty tip box
8	New tip box
9	Prepped library DNA in Eppendorf plate

6 On the SureSelect setup form, under **Select Protocol to Run**, select **Aliquot_Libraries_v1.5.1.pro.**

7 Click **Display Initial Workstation Setup**.

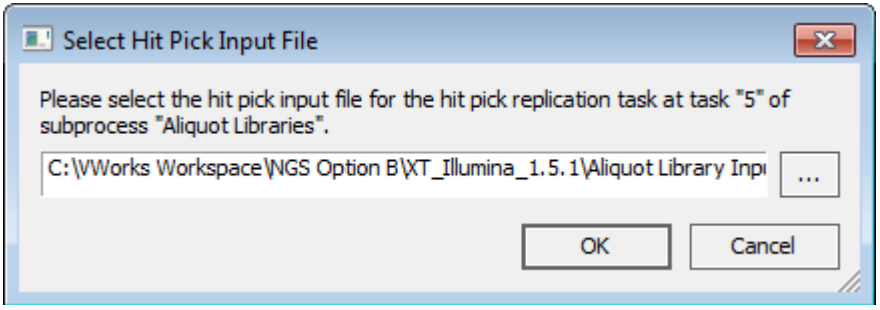


8 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

10 When prompted by the dialog below, browse to the .csv file created for the source plate of the current run, and then click **OK** to start the run.



The library aliquoting protocol takes approximately 1 hour for 96 samples. When complete, the 750-ng samples are in the PCR plate located on Bravo deck position 5.

11 Remove the 750-ng sample plate from the Bravo deck and use a vacuum concentrator to dry the sample at ≤ 45°C.

12 Reconstitute each dried sample with 3.4 µL of nuclease-free water to bring the final concentration to 221 ng/µL. Pipette up and down along the sides of each well for optimal recovery.

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

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

Step 2. Hybridize DNA samples to the probe

In this step, the Agilent NGS Workstation completes the liquid handling steps to prepare for hybridization. Afterward, you transfer the sample plate to a thermal cycler, held at 65°C, to allow hybridization of the prepared DNA samples to one or more Probe Capture Libraries.

Prepare the workstation

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo decks, and BenchCel with a DNA Away decontamination wipe.
3. Turn on the chiller, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Place the silver Nunc DeepWell plate insert on position 6 of the Bravo deck. This insert is required to facilitate heat transfer to DeepWell source plate wells during the Hybridization protocol.

Prepare the SureSelect Block master mix

5 Prepare the appropriate volume of SureSelect Block master mix, on ice, as indicated in Table 50.

Table 50 Preparation of SureSelect Block Master Mix

SureSelect^{XT} 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	6.0 µL	76.5 µL	127.5 µL	178.5 µL	229.5 µL	331.5 µL	663.0 µL
SureSelect Indexing Block 1 (green cap)	2.5 µL	31.9 µL	53.1 µL	74.4 µL	95.6 µL	138.1 µL	276.3 µL
SureSelect Block 2 (blue cap)	2.5 µL	31.9 µL	53.1 µL	74.4 µL	95.6 µL	138.1 µL	276.3 µL
SureSelect ILM Indexing Block 3 (brown cap)	0.6 µL	7.7 µL	12.8 µL	17.9 µL	23.0 µL	33.2 µL	66.3 µL
Total Volume	**11.6 µL**	**147.9 µL**	**246.5 µL**	**345.2 µL**	**443.7 µL**	**640.9 µL**	**1281.9 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 51 to Table 54. Mix the components by pipetting. Keep the master mixes on ice during preparation and aliquoting.

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 51 or Table 52) below.

For runs that use different Probes for individual rows, prepare each master mix as described in Step b (Table 53 or Table 54) below.

a **For runs that use a single Probe for all rows**, prepare the Capture Library Master Mix as listed in Table 51 or Table 52, based on the Mb target size of your design.

Table 51 Preparation of Capture Library Master Mix for target sizes <3.0 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 52 Preparation of Capture Library Master Mix for target sizes >3.0 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 Capture Library Master Mix for each Probe as listed in Table 53 or Table 54, based on the Mb target size of your design. The volumes listed in Table 53 and Table 54 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 53 Preparation of Capture Library Master Mix for target sizes <3.0 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**	**28.9 µL**	**36.3 µL**	**58.6 µL**	**114.4 µL**

Table 54 Preparation of Capture Library Master Mix for target sizes >3.0 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**	**58.6 µL**	**114.4 µL**

Prepare the Hybridization Buffer master mix

7 Prepare the appropriate volume of Hybridization Buffer Master Mix, at room temperature, as indicated in Table 55.

Table 55 Preparation of Hybridization Buffer Master Mix

SureSelect^{XT} Reagent	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 Hyb 1 (orange cap)	140.9 µL	197.3 µL	250.0 µL	310.1 µL	422.8 µL	789.3 µL
SureSelect Hyb 2 (red cap)	5.6 µL	7.9 µL	10.0 µL	12.4 µL	16.9 µL	31.6 µL
SureSelect Hyb 3 (yellow cap or bottle)	56.4 µL	78.9 µL	100.0 µL	124.0 µL	169.1 µL	315.7 µL
SureSelect Hyb 4 (black cap)	73.3 µL	102.6 µL	130.0 µL	161.2 µL	219.9 µL	410.4 µL
Total Volume	**276.2 µL**	**386.7 µL**	**490.0 µL**	**607.7 µL**	**828.7 µL**	**1547 µL**

8 If precipitate forms, warm the hybridization buffer at 65°C for 5 minutes.

Prepare the master mix source plate

9 In a Nunc DeepWell plate, prepare the master mix source plate containing the master mixes prepared in step 5 to step 7 at room temperature. Add the volumes indicated in Table 56 of each master mix to each well of the indicated column of the Nunc DeepWell plate. When using multiple capture libraries in a run, add each Capture Library Master Mix to the appropriate row(s) of the Nunc DeepWell plate. The final configuration of the master mix source plate is shown in Figure 18.

Table 56 Preparation of the Master Mix Source Plate for Hybridization_v1.5.1.pro

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
Block Master Mix	Column 1 (A1-H1)	17.0 µL	29.4 µL	41.7 µL	54.0 µL	78.7 µL	158.8 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)	30.5 µL	44.3 µL	57.2 µL	71.9 µL	99.5 µL	189.3 µL

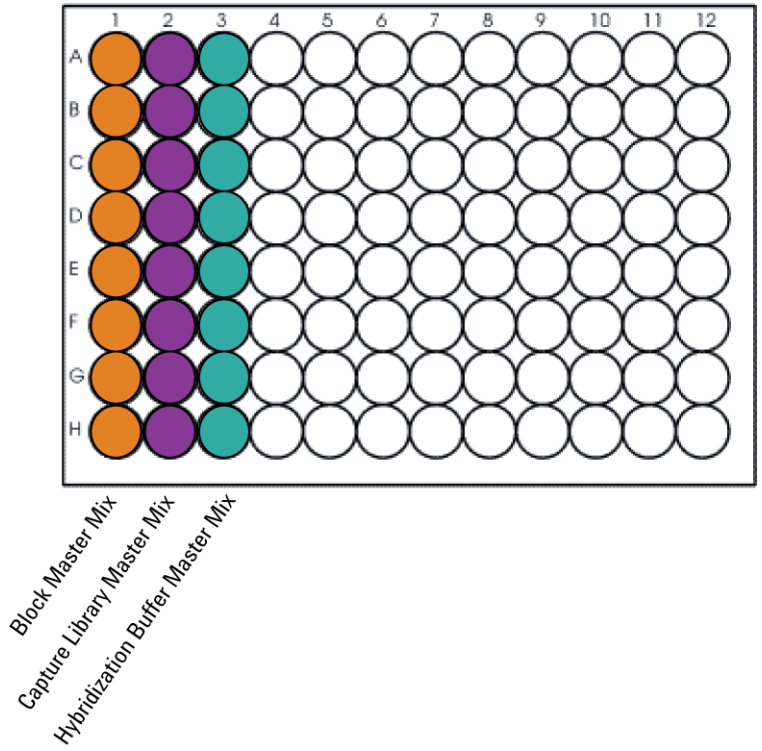


Figure 18 Configuration of the master mix source plate for Hybridization_v1.5.1.pro.

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

11 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles. Keep the master mix plate at room temperature.

Load the Agilent NGS Workstation

12 Load the Labware MiniHub according to Table 57, using the plate orientations shown in Figure 3.

Table 57 Initial MiniHub configuration for Hybridization_v1.5.1.pro

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

13 Load the Bravo deck according to Table 58.

Table 58 Initial Bravo deck configuration for Hybridization_v1.5.1.pro

Location	Content
4	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
5	Empty Eppendorf plate
6	Hybridization Master Mix source plate (unsealed) seated on silver Nunc DeepWell insert
8	Empty tip box
9	750-ng aliquots of prepped gDNA (reconstituted at 221 ng/µL), in Eppendorf plate (unsealed)

14 Load the BenchCel Microplate Handling Workstation according to Table 59.

Table 59 Initial BenchCel configuration for Hybridization_v1.5.1.pro

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

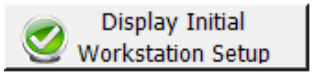
Run VWorks protocol Hybridization_v1.5.1.pro

15 On the SureSelect setup form, under **Select Protocol to Run**, select **Hybridization_v1.5.1.pro.**

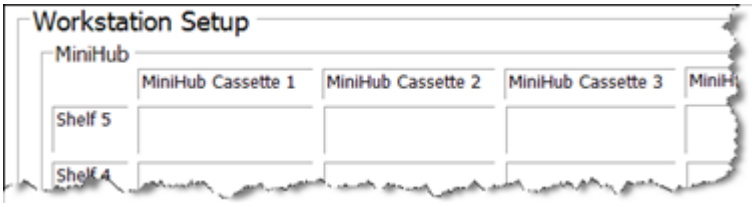
16 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 4 of the Bravo deck.

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

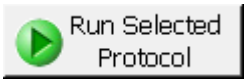
18 Click **Display Initial Workstation Setup**.



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

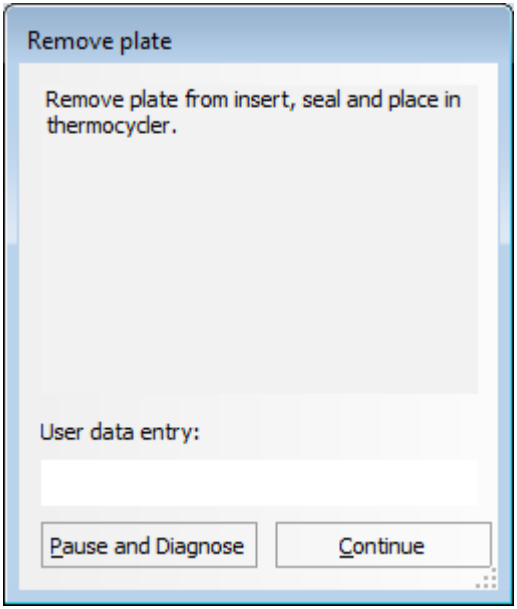


20 When verification is complete, click **Run Selected Protocol**.



The Agilent NGS Workstation transfers SureSelect Block Master Mix to the prepped gDNA-containing wells of the sample plate. When this process is complete, you will be prompted to transfer the plate to the thermal cycler for sample denaturation prior to hybridization.

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



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

23 Transfer the sealed plate to a thermal cycler and run the following program shown in Table 60. After transferring the plate, click **Continue** on the VWorks screen.

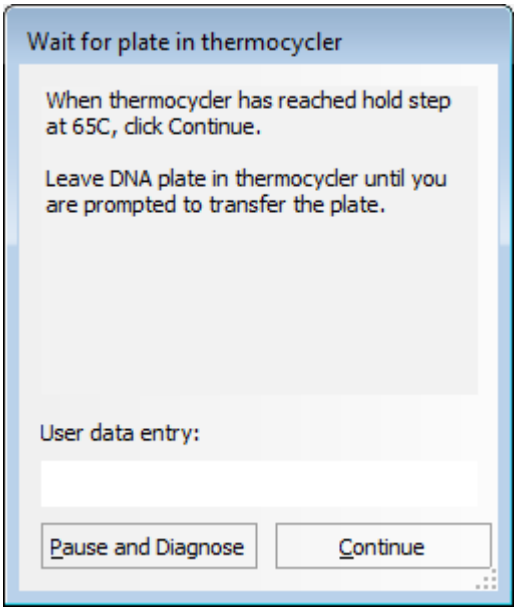
Table 60 Thermal cycler program used for sample denaturation prior to hybridization

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	Hold

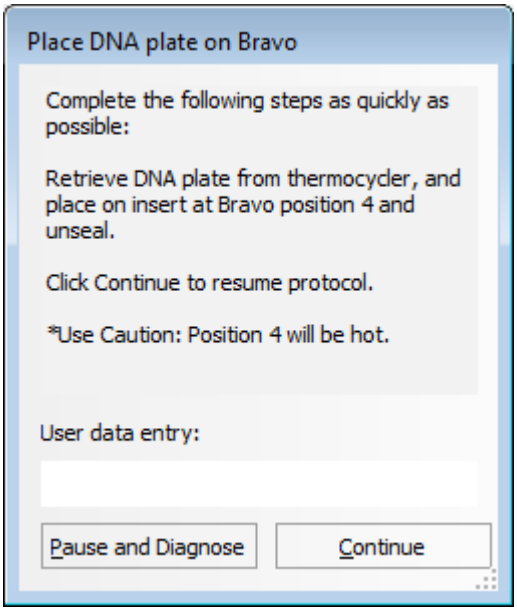
While the sample plate incubates on the thermal cycler, the Agilent NGS Workstation combines aliquots of the Capture Library Master Mix and Hybridization Buffer Master Mix.

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

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



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

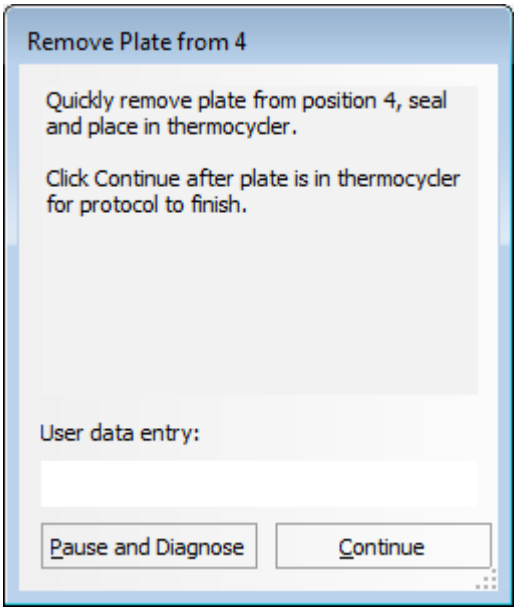


WARNING **Bravo deck position 4 will be hot.**

Use caution when handling components that contact heated deck positions.

The Agilent NGS Workstation transfers the capture library-hybridization buffer mixture to the wells of the PCR plate, containing the mixture of prepped gDNA samples and blocking agents.

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



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

28 Quickly transfer the plate back to the thermal cycler, held at 65°C. After transferring the plate, click **Continue** on the VWorks screen.

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

CAUTION The temperature of the plate in the thermal cycler should be 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.

30 Incubate the hybridization mixture in the thermal cycler for 16 or 24 hours at 65°C with a heated lid at 105°C.

NOTE If you are using the SureCycler 8800 thermal cycler for this step, be sure to set up the incubation using a compression mat over the PCR plate to minimize evaporation.

Step 3. Capture the hybridized DNA

In this step, the gDNA-probe hybrids are captured using streptavidin-coated magnetic beads. This step is run immediately after the 16 or 24-hour hybridization period.

This step is automated by the NGS workstation using the SureSelectCapture&Wash_v1.5.1.rst runset, with a total duration of approximately 3 hours. A workstation operator must be present to complete two actions during the runset, at the time points in the table below. The times provided are approximate; each action is completed in response to a VWorks prompt at the appropriate time in the runset.

Table 61

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

Prepare the workstation

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo decks, and BenchCel with a DNA Away decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 66°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.

Prepare the Dynabeads streptavidin beads

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

5 Wash the magnetic beads.

a In a conical vial, combine the components listed in Table 62. The volumes below include the required overage.

Table 62 Components required for magnetic bead washing procedure

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Dynabeads MyOne Streptavidin T1 bead suspension	50 µL	425 µL	825 µL	1225 µL	1.65 mL	2.5 mL	5.0 mL
SureSelect Binding Buffer	0.2 mL	1.7 mL	3.3 mL	4.9 mL	6.6 mL	10 mL	20 mL
Total Volume	**0.25 mL**	**2.125 mL**	**4.125 mL**	**6.125 mL**	**8.25 mL**	**12.5 mL**	**25 mL**

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

c Put the vial into a magnetic 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.)

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

Table 63 Preparation of magnetic beads for SureSelect Capture&Wash_v1.5.1.rst

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

7 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.

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

Prepare capture and wash solution source plates

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

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

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

12 Place the silver Nunc DeepWell plate insert on position 6 of the Bravo deck. This insert is required to facilitate heat transfer to DeepWell source plate wells during the Capture&Wash runset.

13 Place the *Wash #2* source plate on the insert at position 6 of the Bravo deck. Make sure the plate is seated properly on the silver DeepWell insert.

Load the Agilent NGS Workstation

14 Load the Labware MiniHub according to Table 64, using the plate orientations shown in Figure 3.

Table 64 Initial MiniHub configuration for SureSelect Capture&Wash_v1.5.1.rst

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

15 Load the Bravo deck according to Table 65 (positions 5 and 6 should already be loaded).

Table 65 Initial Bravo deck configuration for SureSelectCapture&Wash_v1.5.1.rst

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

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

Table 66 Initial BenchCel configuration for SureSelectCapture&Wash_v1.5.1.rst

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	2 Tip boxes	Empty	Empty	Empty
2	3 Tip boxes	Empty	Empty	Empty
3	4 Tip boxes	Empty	Empty	Empty
4	5 Tip boxes	Empty	Empty	Empty
6	7 Tip boxes	Empty	Empty	Empty
12	10 Tip boxes	3 Tip boxes	Empty	Empty

Run VWorks runset SureSelectCapture&Wash_v1.5.1.rst

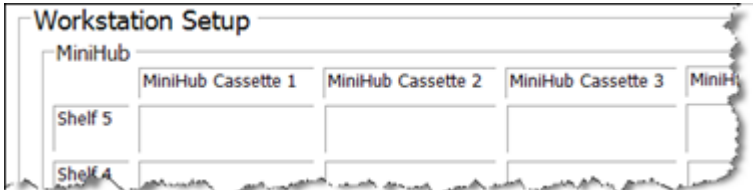
17 On the SureSelect setup form, under **Select Protocol to Run**, select **SureSelectCapture&Wash_v1.5.1.rst.**

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

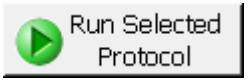
19 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

20 Click **Display Initial Workstation Setup**.

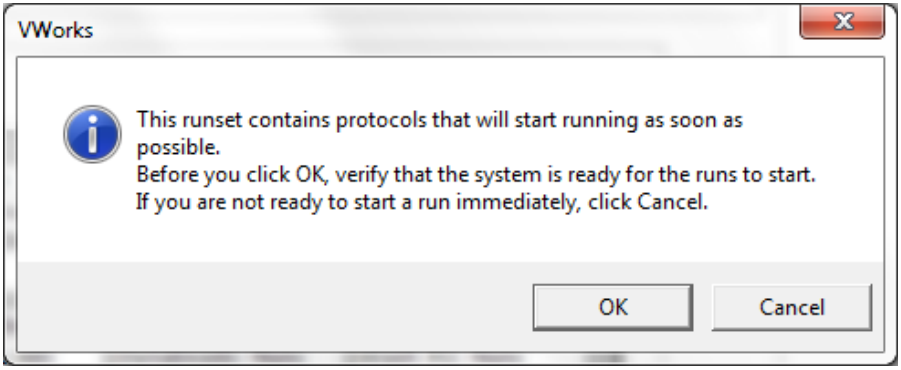
21 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

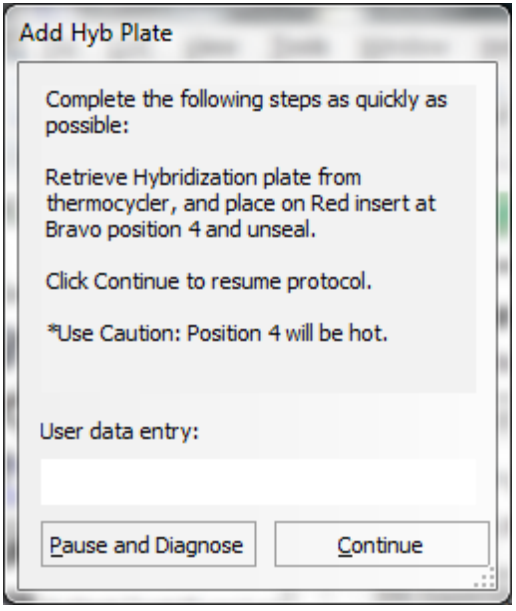


23 When ready to begin the run, click **OK** in the following window. If the temperature of Bravo deck position 4 was not pre-set to 66°C, the runset will pause while position 4 reaches temperature.



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

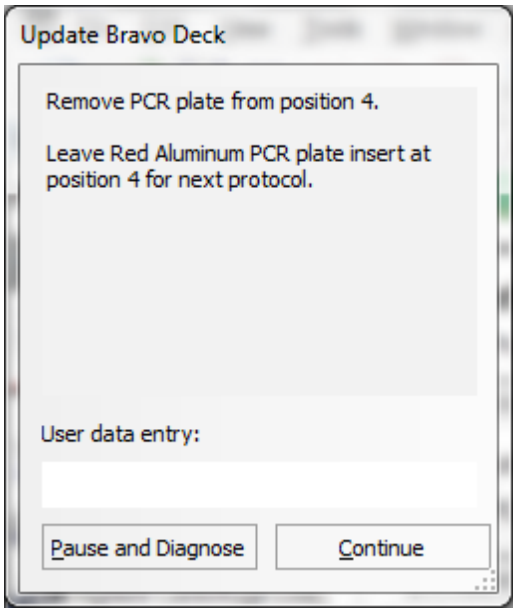
24 When prompted by VWorks as shown below, quickly remove the PCR plate, containing the hybridization reactions held at 65°C, from the thermal cycler. Unseal the plate carefully to avoid splashing, and quickly transfer the plate to position 4 of the Bravo deck, seated in the red insert. Click **Continue**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



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

Use caution when handling components that contact heated deck positions.

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



The remainder of the SureSelectCapture&Wash_v1.5.1.rst runset takes approximately 2 hours. Once the runset is complete, the captured, bead-bound DNA samples are located in the Eppendorf plate at position 9 of the Bravo deck

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

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

6 Indexing

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Step 2. Purify the amplified indexed libraries using AMPure XP beads 136
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This chapter describes the steps to add index tags by amplification, purify, assess quality and quantity of the libraries, and pool indexed samples for multiplexed sequencing.

Step 1. Amplify the captured libraries to add index tags

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

The design size of your Probe Capture Library determines the amplification cycle number used for indexing. Plan your experiments for amplification of samples enriched using probes of similar design sizes on the same plate. See Table 74 on page 135 for cycle number recommendations.

Assign indexes to DNA samples

Select the appropriate indexing primer for each sample.

Use a different index primer for each sample to be sequenced in the same lane. The number of samples that may be combined per lane depends on the sequencing platform performance and the probe design size.

As a guideline, Agilent recommends analyzing 100X amount of sequencing data compared to the Probe Capture Library size for each sample. Specific examples of sequence data requirement recommendations are provided in Table 67. Calculate the number of indexes that can be combined per lane based on these guidelines.

Table 67 Sequencing data requirement guidelines

Probe Size/Description	Recommended Amount of Sequencing Data per Sample*
1 kb up to 0.5 Mb	0.1 to 50 Mb
0.5 Mb up to 2.9 Mb	50 to 290 Mb
3 Mb up to 5.9 Mb	300 to 590 Mb
6 Mb up to 11.9 Mb	600 to 1190 Mb
12 Mb up to 24 Mb	1.2 to 2.4 Gb
Human All Exon v5	4 Gb
Human All Exon v5 + UTRs	6 Gb
Human All Exon 50 Mb	5 Gb
Human DNA Kinome	320 Mb
Mouse All Exon	5 Gb

* Agilent recommends analyzing 100X amount of sequencing data compared to the Probe size for each sample. Pool samples according to your expected sequencing output.

Prepare the workstation

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

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

3 Pre-set the temperature of Bravo deck positions 4 and 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. Bravo deck position 4 corresponds to CPAC 2, position 1 and Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.

Prepare indexing primers and PCR master mix

CAUTION Do not use amplification enzymes other than Herculase II Fusion DNA Polymerase. Other enzymes have not been validated.

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.

4 Prepare the indexing primers in the PCR plate to be used for the amplification automation protocol. In each well of the PCR plate, combine 5 µL of the specific indexing primer assigned to the sample well with 4 µL of water. Keep the plate on ice.

5 Prepare the appropriate volume of PCR master mix, according to Table 68. Mix well using a vortex mixer and keep on ice.

Table 68 Preparation of PCR Master Mix for Post-CaptureIndexing_XT_ILM_v1.5.1.pro

SureSelect^{XT} 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	14.5 µL	184.9 µL	308.1 µL	431.4 µL	554.6 µL	801.1 µL	1602.3 µL
Herculase II 5X Reaction Buffer*	10.0 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1105.0 µL
SureSelect Indexing Post-Capture PCR (Forward) Primer†	1.0 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
dNTP mix*	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	27.6 µL	55.3 µL
Herculase II polymerase	1.0 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
Total Volume	**27.0 µL**	**344.3 µL**	**573.8 µL**	**803.4 µL**	**1032.8 µL**	**1491.8 µL**	**2983.5 µL**

* Included with the Herculase II Fusion DNA Polymerase. *Do not use the buffer or dNTP mix from any other kit.*

† Included in SureSelect XT Automation ILM Module Box 2.

6 Using the same Nunc DeepWell master mix source plate that was used for the Hybridization_v1.5.1.pro protocol, add the volume of PCR master mix indicated in Table 69 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 19.

Table 69 Preparation of the Master Mix Source Plate for Post-CaptureIndexing_XT_ILM_v1.5.1.pro

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
PCR Master Mix	Column 4 (A4-H4)	39.7 µL	68.3 µL	97.0 µL	125.7 µL	183.1 µL	369.6 µL

NOTE If you are using a new DeepWell plate for the post-capture PCR source plate (for example, when amplifying the second half of the captured DNA sample), leave columns 1 to 3 empty and add the PCR Master Mix to column 4 of the new plate.

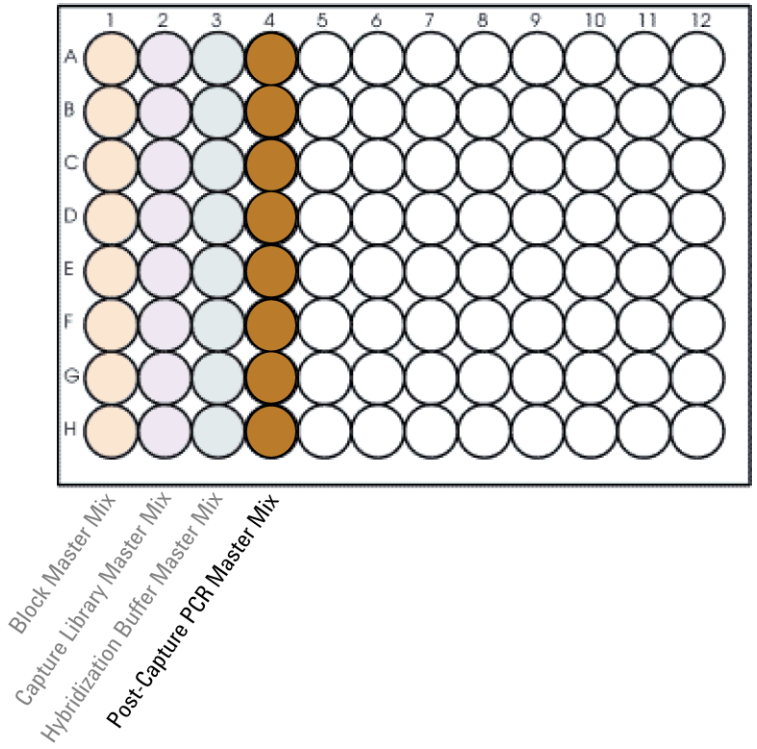


Figure 19 Configuration of the master mix source plate for Post-CaptureIndexing_ XT_ILM_v1.5.1.pro. Columns 1–3 were used to dispense master mixes for the Hybridization_v1.5.1.pro protocol.

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.

Load the Agilent NGS Workstation

9 Load the Labware MiniHub according to Table 70, using the plate orientations shown in Figure 3.

Table 70 Initial MiniHub configuration for Post-CaptureIndexing_XT_ILM_v1.5.1.pro

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

10 Load the Bravo deck according to Table 71.

Table 71 Initial Bravo deck configuration for Post-CaptureIndexing_XT_ILM_v1.5.1.pro

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

11 Load the BenchCel Microplate Handling Workstation according to Table 72.

Table 72 Initial BenchCel configuration for Post-CaptureIndexing_XT_ILM_v1.5.1.pro

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

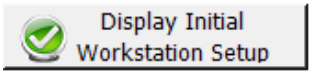
Run VWorks protocol Post-CaptureIndexing_XT_ILM_v1.5.1.pro

12 On the SureSelect setup form, under **Select Protocol to Run**, select **Post- CaptureIndexing_XT_ILM_v1.5.1.pro.**

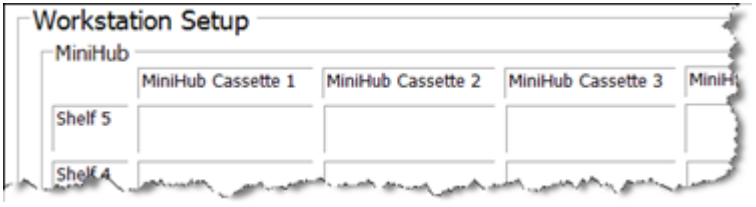
13 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

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

15 Click **Display Initial Workstation Setup**.



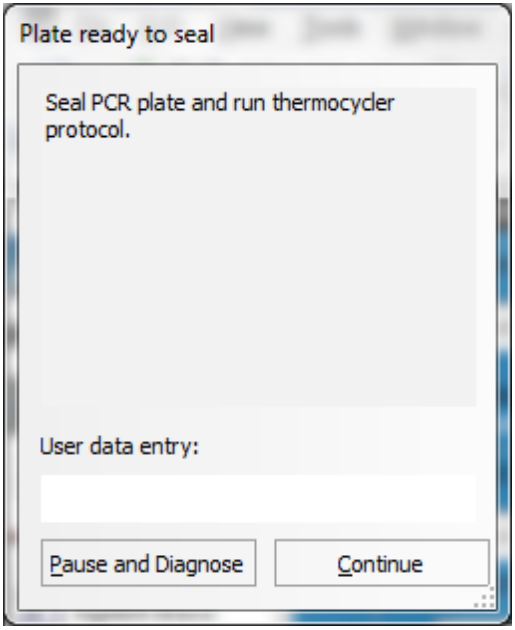
16 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

Running the Post-CaptureIndexing_XT_ILM_v1.5.1.pro protocol takes approximately 15 minutes. Once complete, the PCR-ready samples, containing captured DNA and PCR master mix, are located in the PCR plate at position 6 of the Bravo deck. The Eppendorf plate containing the remaining bead-bound captured DNA samples, which may be stored for future use at 4°C overnight, or at –20°C for longer-term storage, is located at position 4 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 Transfer the PCR plate to a thermal cycler and run the PCR amplification program shown in Table 73 using the cycle number specified in Table 74.

Table 73 Post-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10 to 16 Cycles see Table 74 for recommendations based on probe design size	98°C	30 seconds
		57°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

Table 74 Post-capture PCR recommended cycle number

Probe Size/Description	Cycles
<0.5 Mb	16 cycles
0.5 to 1.49 Mb	14 cycles
> 1.5 Mb	12 cycles
All Exon and Exome probes	10 to 12 cycles
OneSeq Constitutional Research Panel	10 cycles
OneSeq Hi Res CNV Backbone-based custom designs	10 cycles
OneSeq 1Mb CNV Backbone-based custom designs	10 to 12 cycles

NOTE

Amplify the captured DNA using a minimal number of PCR cycles. If yield is too low or non-specific high molecular weight products are observed, adjust the number of cycles accordingly with the remaining captured DNA template.

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

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

Prepare the workstation and reagents

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo decks, and BenchCel with a Nucleoclean decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
5. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
6. Prepare a Nunc DeepWell source plate containing AMPure XP beads. For each well to be processed, add 95 µL of homogeneous AMPure XP beads per well to the Nunc DeepWell plate.
7. Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water.
8. Prepare a separate Thermo Scientific reservoir containing 45 mL of freshly-prepared 70% ethanol.

9 Load the Labware MiniHub according to Table 75, using the plate orientations shown in Figure 3.

Table 75 Initial MiniHub configuration for AMPureXP_XT_ILM_v1.5.1.pro:Post-Capture PCR

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	Empty	Empty	Empty
Shelf 4	Empty	Empty	Empty	Empty
Shelf 3	Empty	Empty Eppendorf Plate	Empty	Empty
Shelf 2	Empty	Nuclease-free water reservoir from step 7	AMPure XP beads in Nunc DeepWell plate from step 6	Empty
Shelf 1 (Bottom)	Empty	70% ethanol reservoir from step 8	Empty	Empty tip box

10 Load the Bravo deck according to Table 76.

Table 76 Initial Bravo deck configuration for AMPureXP_XT_ILM_v1.5.1.pro:Post-Capture PCR

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

11 Load the BenchCel Microplate Handling Workstation according to Table 77.

Table 77 Initial BenchCel configuration for AMPureXP_XT_ILM_v1.5.1.pro:Post-Capture PCR

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

Run VWorks protocol AMPureXP_XT_ILM_v1.5.1.pro:Post-Capture PCR

12 On the SureSelect setup form, under **Select Protocol to Run**, select **AMPureXP_XT_ILM_v1.5.1.pro:Post- Capture PCR**.

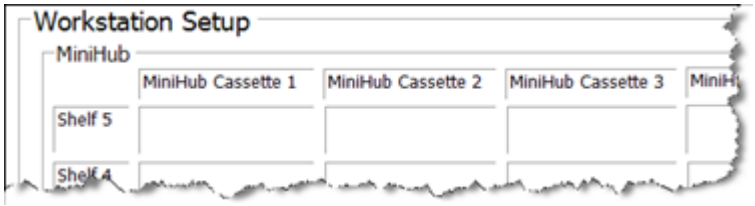
NOTE AMPureXP purification protocols are used during multiple steps of the SureSelect automation workflow. Be sure to select the correct workflow step when initiating the automation protocol.

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

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

15 Click **Display Initial Workstation Setup**.

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



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

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

Step 3. Assess indexed DNA quality

Option 1: Analysis using the 2100 Bioanalyzer and High Sensitivity DNA Assay

Use the Bioanalyzer High Sensitivity DNA Assay to analyze the amplified captured DNA. Perform the assay according to the High Sensitivity DNA Kit Guide.

1 Set up the 2100 Bioanalyzer instrument as instructed in the reagent kit guide.

NOTE Version B.02.07 or higher of the Agilent 2100 Expert Software is required for High Sensitivity DNA Assay Kit runs.

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

3 Vortex the plate to mix samples in each well, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal.

4 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.

NOTE For some samples, Bioanalyzer results are improved by diluting 1 µL of the sample in 9 µL of 10 mM Tris, 1 mM EDTA prior to analysis. Be sure to mix well by vortexing at 2000 rpm on the IKA vortex supplied with the Bioanalyzer before analyzing the diluted samples.

5 Load the prepared chip into the instrument and start the run within five minutes after preparation.

6 Verify that the electropherogram shows the peak of DNA fragment size positioned between 250 to 350 bp. A sample electropherogram is shown in Figure 20.

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.

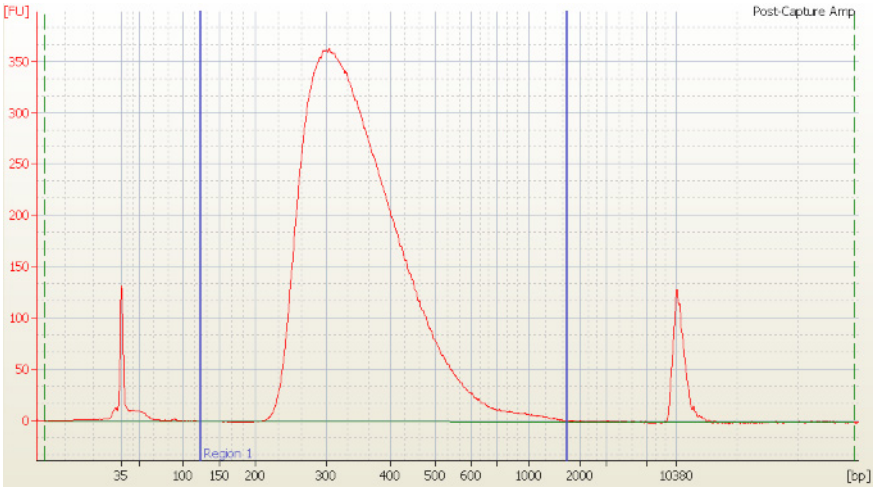


Figure 20 Analysis of indexed DNA using the High Sensitivity DNA Assay.

Option 2: Analysis using an Agilent TapeStation and High Sensitivity D1000 ScreenTape

Use a High Sensitivity D1000 ScreenTape and associated reagent kit to analyze the amplified captured DNA. Perform the assay according to the Agilent High Sensitivity D1000 Assay Quick Guide.

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

2 Vortex the plate to mix samples in each well, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal.

3 Prepare the TapeStation samples as instructed in the reagent kit guide. Use 2 µL of each DNA sample diluted with 2 µL of High Sensitivity D1000 sample buffer for the analysis.

CAUTION For accurate quantitation, make sure to thoroughly mix the combined DNA and sample buffer by vortexing the assay plate or tube strip for 1 minute on the IKA MS3 vortex mixer provided with the 4200/4150 TapeStation system before loading the samples.

4 Load the sample plate or tube strips from step 3, the High Sensitivity D1000 ScreenTape, and loading tips into the TapeStation as instructed in the reagent kit guide. Start the run.

5 Verify that the electropherogram shows the peak of DNA fragment size positioned between 250 to 350 bp. A sample electropherogram is shown in Figure 21.

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

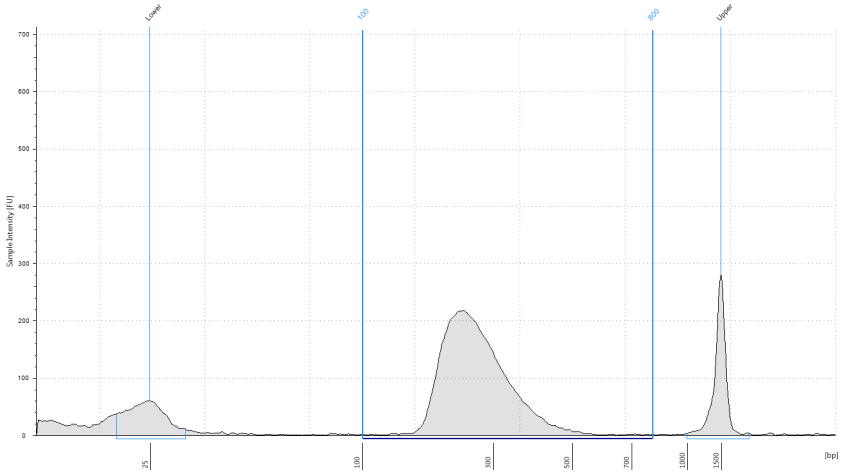


Figure 21 Analysis of indexed DNA using a High Sensitivity D1000 ScreenTape.

Step 4. Quantify each index-tagged library by QPCR

Refer to the protocol that is included with the Agilent QPCR NGS Library Quantification Kit (p/n G4880A) for more details to do this step.

1. Use the Agilent QPCR NGS Library Quantification Kit (for Illumina) to determine the concentration of each index-tagged captured library.
2. Prepare a standard curve using the quantification standard included in the kit, according to the instructions provided in the user guide.
3. Dilute each index-tagged captured library such that it falls within the range of the standard curve.

   Typically this corresponds to approximately a 1:1000 to 1:10,000 dilution of the captured DNA.
4. Prepare the QPCR master mix with Illumina adaptor-specific PCR primers according to instructions provided in the kit.
5. Add an aliquot of the master mix to PCR tubes and add template.
6. On a QPCR system, such as the Mx3005p, run the thermal profile outlined in the QPCR NGS Library Quantification kit user guide. Use the SYBR Green instrument setting.
7. Use the standard curve to determine the concentration of each unknown index-tagged library, in nM.

   The concentration will be used to accurately pool samples for multiplexed sequencing.

NOTE

In most cases, the cycle numbers in Table 74 will produce an adequate yield for sequencing without introducing bias or non-specific products. If yield is too low or non-specific products are observed, adjust the number of cycles accordingly with the remaining captured DNA template.

Step 5. Pool samples for Multiplexed Sequencing

1 Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool. For each library, use the formula below to determine the amount of indexed sample to use.

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

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

is the number of indexes, and

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

Table 78 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.

Table 78 Example of indexed sample volume calculation for total volume of 20 µL

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

2 Adjust the final volume of the pooled library to the desired final concentration.

- If the final volume of the combined index-tagged samples is less than the desired final volume, V(f), add Low TE to bring the volume to the desired level.
- If the final volume of the combined index-tagged samples is greater than the final desired volume, V(f), lyophilize and reconstitute to the desired volume.

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

Guidelines for sequencing sample preparation and run setup

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See Table 79 for kit configurations compatible with the recommended read length.

The optimal seeding concentration for SureSelect^{XT} target-enriched libraries varies according to sequencing platform, run type, and Illumina kit version. See Table 79 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 79.

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

Table 79 Illumina Kit Configuration and Seeding Concentration 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	200 Cycle Kit	v3	9–10 pM
HiSeq 2500	High Output	2 × 100 bp	250 Cycle Kit	v4	12–14 pM
HiSeq 2000	All Runs	2 × 100 bp	200 Cycle Kit	v3	6–9 pM
HiSeq 2000	All Runs	2 × 100 bp	250 Cycle Kit	v4	8–12 pM
MiSeq	All Runs	2 × 100 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	300 Cycle Kit	v2.5	1.2–1.5 pM
HiSeq 3000/4000	All Runs	2 × 100 bp	300 Cycle Kit	v1	230–240 pM
NovaSeq 6000	Standard Workflow Runs	2 × 100 bp	300 Cycle Kit	v1	300–500 pM
NovaSeq 6000	Xp Workflow Runs	2 × 100 bp	300 Cycle Kit	v1	200–400 pM

Sequencing run setup guidelines

Sequencing runs must be set up to perform an 8-bp index read. For complete index sequence information, see the Table 87 on page 153.

For the HiSeq platform, **Cycles** settings can be specified on the *Run Configuration* screen of the instrument control software interface after choosing *Custom* from the **Index Type** selection buttons. Use the **Cycles** settings shown in Table 80.

For the NextSeq and NovaSeq platforms, the **Read Length** settings shown in Table 80 can be specified on the *Run Setup* screen of the instrument control software interface. In the **Custom Primers** section of the NextSeq or NovaSeq platform *Run Setup* screen, clear (do **not** select) the checkboxes for all primers (*Read 1, Read 2, Index 1* and *Index 2*).

Table 80 Cycle Number settings for HiSeq/NextSeq/NovaSeq platforms

Run Segment	Cycles/Read Length
Read 1	100
Index 1 (i7)	8
Index 2 (i5)	0
Read 2	100

For the MiSeq platform, use the Illumina Experiment Manager (IEM) software to generate a Sample Sheet that includes the run parameters specified in Table 81.

Table 81 Run parameters for MiSeq platform Sample Sheet

Parameter	Entry
Workflow	GenerateFASTQ
Cycles for Read 1	100 for v2 chemistry 75 for v3 chemistry
Cycles for Read 2	100 for v2 chemistry 75 for v3 chemistry
Index 1 (i7) Sequence (enter in Data Section for each sample)	Type the 8-nt index sequence for each individual sample (see Table 87 on page 153).

7 Reference

Kit Contents 150
Nucleotide Sequences of SureSelect^{XT} 8-bp Indexes 153

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

Kit Contents

SureSelect^{XT} Automation Reagent Kits contain the component kits listed in Table 82. The contents of each component kit are detailed in Table 83 through Table 85.

Table 82 SureSelect^{XT} Automation Reagent Kit Contents

Product	Storage Condition	96 Reactions	480 Reactions
SureSelect XT Library Prep Kit ILM	–20°C	5500-0133	5 x 5500-0133
SureSelect Target Enrichment Box 1	Room Temperature	5190-8646	5 x 5190-8646
SureSelect XT Automation ILM Module Box 2	–20°C	5190-3730	5190-3732

Table 83 SureSelect XT Library Prep Kit ILM Content

Kit Component	Format
10X End Repair Buffer	tube with clear cap
10X Klenow Polymerase Buffer	tube with blue cap
5X T4 DNA Ligase Buffer	tube with green cap
T4 DNA Ligase	tube with red cap
Exo(–) Klenow	tube with red cap
T4 DNA Polymerase	tube with purple cap
Klenow DNA Polymerase	tube with yellow cap
T4 Polynucleotide Kinase	tube with orange cap
dATP	tube with green cap
dNTP Mix	tube with green cap
SureSelect Adaptor Oligo Mix	tube with brown cap
SureSelect Primer (forward primer)	tube with brown cap
SureSelect^{XT} Indexes, 8 bp reverse primers*	SureSelect 8bp Indexes A01 through H12, provided in blue 96-well plate†

* See Table 87 on page 153 for index sequences.

† See Table 86 on page 152 for a plate map.

Table 84 SureSelect Target Enrichment Box 1 Content

Kit Component	Format
SureSelect Hyb 1	tube with orange cap
SureSelect Hyb 2	tube with red cap
SureSelect Hyb 4	tube with black cap
SureSelect Binding Buffer	bottle
SureSelect Wash Buffer 1	bottle
SureSelect Wash Buffer 2	bottle

Table 85 SureSelect XT Automation ILM Module Box 2 Content

Kit Component	96 Reactions	480 Reactions
SureSelect Hyb 3	tube with yellow cap	bottle
SureSelect Indexing Block 1	tube with green cap	tube with green cap
SureSelect Block 2	tube with blue cap	tube with blue cap
SureSelect ILM Indexing Block 3	tube with brown cap	tube with brown cap
SureSelect RNase Block	tube with purple cap	tube with purple cap
SureSelect ILM Indexing Pre-Capture PCR Reverse Primer	tube with clear cap	tube with clear cap
SureSelect ILM Indexing Post-Capture Forward PCR Primer	tube with orange cap	tube with orange cap

Table 86 Plate map for SureSelect 8bp Indexes A01 through H12, provided in blue plate in Library Prep kit p/n 5500-0133

	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
C	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
D	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
E	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
F	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
H	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

Nucleotide Sequences of SureSelect^{XT} 8-bp Indexes

Each index is 8 nt in length. See page 147 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 87 SureSelect^{XT} Indexes, for indexing primers provided in blue 96-well plate

Index	Sequence	Index	Sequence	Index	Sequence	Index	Sequence
A01	ATGCCTAA	A04	AACTCACC	A07	ACGTATCA	A10	AATGTTGC
B01	GAATCTGA	B04	GCTAACGA	B07	GTCTGTCA	B10	TGAAGAGA
C01	AACGTGAT	C04	CAGATCTG	C07	CTAAGGTC	C10	AGATCGCA
D01	CACTTCGA	D04	ATCCTGTA	D07	CGACACAC	D10	AAGAGATC
E01	GCCAAGAC	E04	CTGTAGCC	E07	CCGTGAGA	E10	CAACCACA
F01	GACTAGTA	F04	GCTCGGTA	F07	GTGTTCTA	F10	TGGAACAA
G01	ATTGGCTC	G04	ACACGACC	G07	CAATGGAA	G10	CCTCTATC
H01	GATGAATC	H04	AGTCACTA	H07	AGCACCTC	H10	ACAGATTC
A02	AGCAGGAA	A05	AACGCTTA	A08	CAGCGTTA	A11	CCAGTTCA
B02	GAGCTGAA	B05	GGAGAACA	B08	TAGGATGA	B11	TGGCTTCA
C02	AAACATCG	C05	CATCAAGT	C08	AGTGGTCA	C11	CGACTGGA
D02	GAGTTAGC	D05	AAGGTACA	D08	ACAGCAGA	D11	CAAGACTA
E02	CGAACTTA	E05	CGCTGATC	E08	CATACCAA	E11	CCTCCTGA
F02	GATAGACA	F05	GGTGCGAA	F08	TATCAGCA	F11	TGGTGGTA
G02	AAGGACAC	G05	CCTAATCC	G08	ATAGCGAC	G11	AACAACCA
H02	GACAGTGC	H05	CTGAGCCA	H08	ACGCTCGA	H11	AATCCGTC
A03	ATCATTCC	A06	AGCCATGC	A09	CTCAATGA	A12	CAAGGAGC
B03	GCCACATA	B06	GTACGCAA	B09	TCCGTCTA	B12	TTCACGCA
C03	ACCACTGT	C06	AGTACAAG	C09	AGGCTAAC	C12	CACCTTAC
D03	CTGGCATA	D06	ACATTGGC	D09	CCATCCTC	D12	AAGACGGA
E03	ACCTCCAA	E06	ATTGAGGA	E09	AGATGTAC	E12	ACACAGAA
F03	GCGAGTAA	F06	GTCGTAGA	F09	TCTTCACA	F12	GAACAGGC
G03	ACTATGCA	G06	AGAGTCAA	G09	CCGAAGTA	G12	AACCGAGA
H03	CGGATTGC	H06	CCGACAAC	H09	CGCATACA	H12	ACAAGCTA

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In This Book

This guide contains information to run the SureSelect^{XT} Automated Target Enrichment for the Illumina Platform protocol using a SureSelect^{XT} Automated Reagent Kit and automation protocols provided with the Agilent NGS Workstation Option B.

Version N0, November 2020

G7550-90000

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