SureSelect^{XT} Automated Target Enrichment for Illumina Paired-End Multiplexed Sequencing

Automated using Agilent NGS Workstation Option B

Protocol

Version K0, August 2017

SureSelect platform manufactured with Agilent SurePrint Technology

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

Agilent Technologies

Notices

Manual Part Number

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Acknowledgement

Technical Support

For technical product support, contact your local Agilent Support Services representative. For Agilent's worldwide sales and support center telephone numbers, go to www.agilent.com/chem/contactus

or send an email to:
SureSelect.Support@agilent.com

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Safety Notices

CAUTION

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A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

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 K0

- Support for VWorks software version 13.1.0.1366 and Agilent NGS Workstation Option B p/n G5574AA (see Table 4 on page 15)
- Updates to downstream sequencing instructions including sequencing kit selection and seeding concentration guidelines (see page 150)
- Updates to Agilent 2100 Bioanalyzer system ordering information (see Table 4 on page 16)
- Addition of Agilent 4200 Tapestation system-compatible plasticware ordering information (see Table 4 on page 16)
- Updates to reference information for Agilent NGS Workstation component user guides (see Table 5 on page 18)

What's New in Version J0

- Support for Clinical Research Exome V2 Capture Libraries (see Table 3 on page 14).
- Removal of reference information for obsolete kits containing 6-bp indexing primers 1–16, provided in clear-capped tubes (typically received before February 2015). If you need assistance with kits containing these obsolete indexing primer components, please contact Technical Support.
- Support for VWorks software version 13.0.0.1360 (see Table 4 on page 15)
- Updates to supplier name for materials purchased from Thermo Fisher Scientific (see Table 1 on page 12 and Table 4 on page 15)
- Kit use-by information removed. See product labels and the Certificate of Analysis for each component for expiration date information.

Content

1 Before You Begin

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

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 35) 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 40.
- 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 Capture Library	Select the appropriate library from Table 2 or Table 3
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)	Ambion Cat #AM9930
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
Agencourt AMPure XP Kit	Beckman Coulter Genomics
60 mL	p/n A63881
450 mL	p/n A63882
Quant-iT dsDNA BR Assay Kit, for use with the Qubit fluorometer	Thermo Fisher Scientific
100 assays, 2-1000 ng	Cat #Q32850
500 assays, 2-1000 ng	Cat #Q32853
Dynabeads MyOne Streptavidin T1	Thermo Fisher Scientific
2 mL	Cat #65601
10 mL	Cat #65602
100 mL	Cat #65603
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 SureSelect^{XT} Automation Capture Libraries

Capture Library	96 Reactions	480 Reactions
SureSelect^{XT} Clinical Research Exome V2	5190-9493	–
SureSelect^{XT} Clinical Research Exome V2 Plus 1	5190-9496	–
SureSelect^{XT} Clinical Research Exome V2 Plus 2	5190-9499	–
SureSelect^{XT} Clinical Research Exome	5190-7344	–
SureSelect^{XT} Focused Exome	5190-7789	–
SureSelect^{XT} Focused Exome Plus 1	5190-7792	–
SureSelect^{XT} Focused Exome Plus 2	5190-7796	–
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} Human All Exon v6 Plus 1	5190-8868	–
SureSelect^{XT} Human All Exon v6 Plus 2	5190-8871	–
SureSelect^{XT} Human All Exon v5	5190-6210	–
SureSelect^{XT} Human All Exon v5 + UTRs	5190-6215	–
SureSelect^{XT} Human All Exon v5 + lncRNA	5190-6448	–
SureSelect^{XT} Human All Exon v5 Plus	5190-6224	–
SureSelect^{XT} Human All Exon v4	5190-4633	5190-4635
SureSelect^{XT} Human All Exon v4 + UTRs	5190-4638	5190-4640
SureSelect^{XT} Mouse All Exon	5190-4643	5190-4645
SureSelect^{XT} Custom 1 kb up to 499 kb (reorder)	5190-4808 (5190-4813)	5190-4810 (5190-4815)
SureSelect^{XT} Custom 0.5 Mb up to 2.9 Mb (reorder)	5190-4818 (5190-4823)	5190-4820 (5190-4825)
SureSelect^{XT} Custom 3 Mb up to 5.9 Mb (reorder)	5190-4828 (5190-4833)	5190-4830 (5190-4835)
SureSelect^{XT} Custom 6 Mb up to 11.9 Mb (reorder)	5190-4838 (5190-4843)	5190-4840 (5190-4845)
SureSelect^{XT} Custom 12 Mb up to 24 Mb (reorder)	5190-4898 (5190-4903)	5190-4900 (5190-4905)

Table 3 Compatible ClearSeq and OneSeq Automation Capture Libraries

Capture Library	96 Reactions	480 Reactions
ClearSeq Comprehensive Cancer XT	5190-8013	–
ClearSeq Comprehensive Cancer Plus XT	5190-8016	–
ClearSeq Inherited Disease XT	5190-7520	–
ClearSeq Inherited Disease Plus XT	5190-7523	–
ClearSeq DNA Kinome XT	5190-4648	5190-4650
OneSeq 1Mb CNV Backbone + Custom 1–499 kb	5190-9464	
OneSeq 1Mb CNV Backbone + Custom 0.5–2.9 Mb	5190-9467	
OneSeq 1Mb CNV Backbone + Custom 3–5.9 Mb	5190-9470	
OneSeq 1Mb CNV Backbone + Custom 6–11.9 Mb	5190-9473	
OneSeq 1Mb CNV Backbone + Custom 12–24 Mb	5190-9476	
OneSeq Constitutional Research Panel	5190-8704	–
OneSeq Hi Res CNV Backbone + Custom 1–499 kb	5190-8888	–
OneSeq Hi Res CNV Backbone + Custom 0.5 –2.9 Mb	5190-8891	–
OneSeq Hi Res CNV Backbone + Custom 3–5.9 Mb	5190-8894	–
OneSeq Hi Res CNV Backbone + Custom 6–11.9 Mb	5190-8897	–

Required Equipment

Table 4 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	SureCycler 8800 Thermal Cycler (Agilent p/n G8810A), 96 well plate module (Agilent p/n G8810A) and compression mats (Agilent p/n 410187) or equivalent
PCR plates compatible with selected Thermal Cycler, e.g. Agilent semi-skirted PCR plate for the SureCycler 8800 Thermal Cycler When selecting plates for another thermal cycler, see Table 10 on page 34 for the list of PCR plates supported in automation protocols	Agilent p/n 401334
Eppendorf twin.tec full-skirted 96-well PCR plates	Eppendorf p/n 951020401 or 951020619
Thermo Scientific Reservoirs	Thermo Scientific p/n 1064156
Nunc DeepWell Plates, sterile, 1.3-mL well volume	Thermo Scientific p/n 260251
Axygen 96 Deep Well Plate, 2 mL, Square Well (waste reservoirs; working volume 2.2 mL)	Axygen p/n P-2ML-SQ-C E & K Scientific p/n EK-2440
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Qubit Fluorometer	Thermo Fisher Scientific p/n Q32857
Qubit assay tubes	Thermo Fisher Scientific p/n Q32856
Covaris Sample Preparation System, S-series or E-series model	Covaris
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
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vacuum concentrator	Savant SpeedVac, model DNA120, with 96-well plate rotor, model RD2MP, or equivalent

Table 4 Required Equipment (continued)

Description	Vendor and part number
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	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Vortex mixer	
Timer	
DNA Analysis Platform and Consumables	
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
OR	
Agilent 4200 TapeStation*	Agilent p/n G2991AA
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
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 2200 TapeStation, p/n G2964AA or G2965AA. ScreenTape devices and associated reagents listed in this table are compatible with both platforms.

2 Using the Agilent NGS Workstation for SureSelect Target Enrichment

This chapter contains an orientation to the Agilent NGS Workstation, an overview of the SureSelect^{XT} 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 5.

Review the user guides listed in Table 5 (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 5 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.

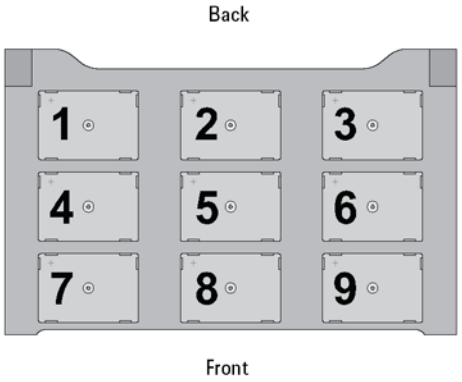


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

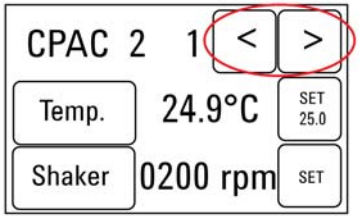
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

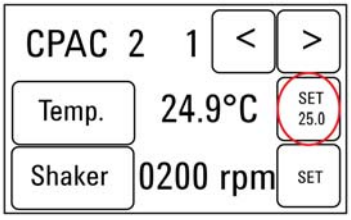
Table 6 Inheco Multi TEC Control touchscreen designations

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

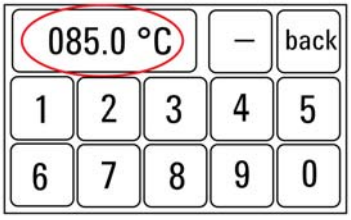
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



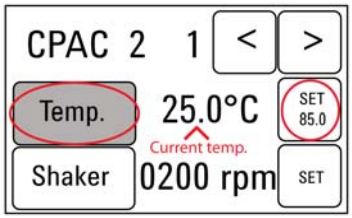
2 To set the temperature of the selected block, press the SET button.



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



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



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

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

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

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

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

3 Press the **START** button.

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

VWorks Automation Control Software

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

NOTE

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

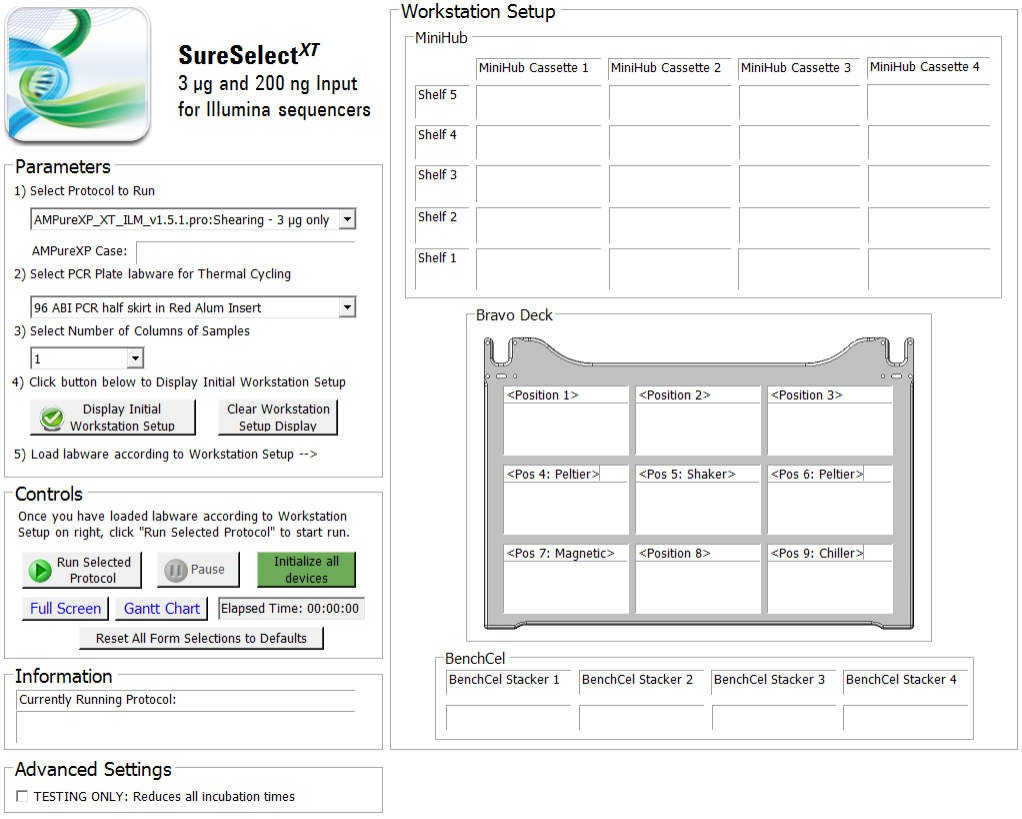
1. Double-click the VWorks icon or the XT_ILM_v1.5.1.VWForm shortcut on the Windows desktop to start the VWorks software.
2. If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
3. In the User Authentication dialog, type your VWorks user name and password, and 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

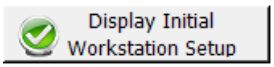
Use the VWorks form SureSelectXT_ILM_v1.5.1.VWForm, shown below, to set up and start each SureSelect automation protocol or runset.



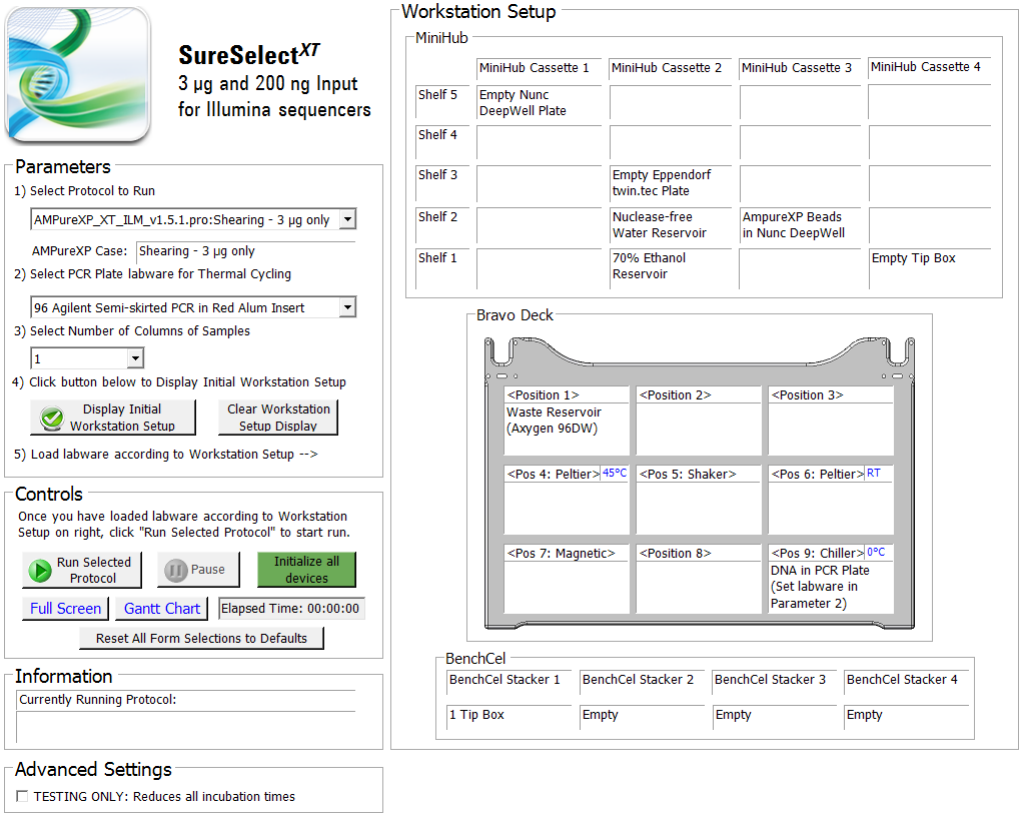
1 Open the form using the XT_ILM_v1.5.1.VWForm shortcut on your desktop.

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.

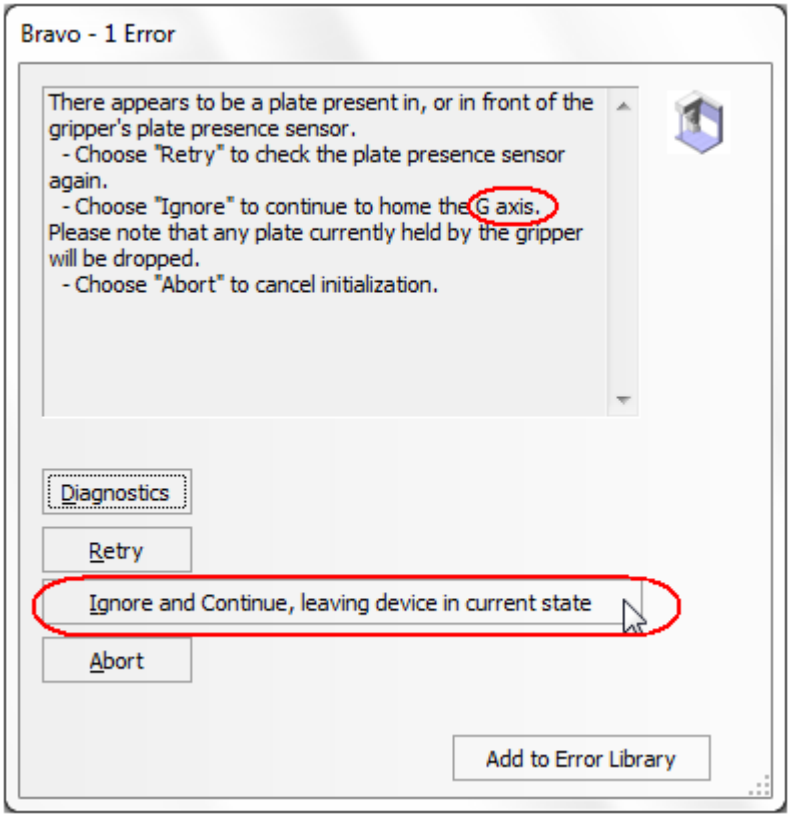


5 After verifying that the NGS Workstation has been set up correctly, click **Run Selected Protocol**.

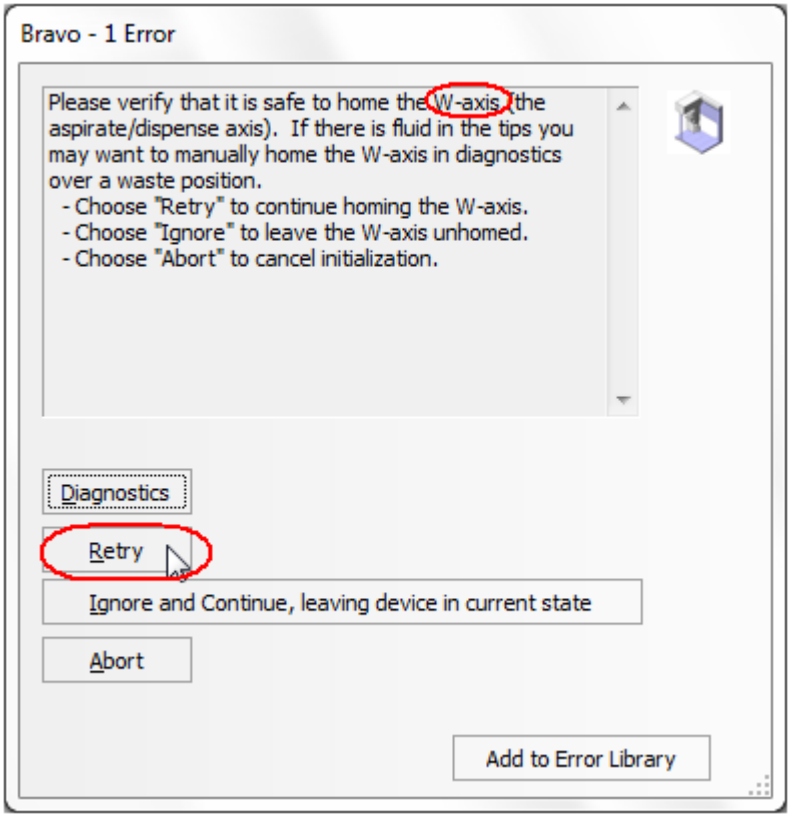
Error messages encountered at start of run

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.

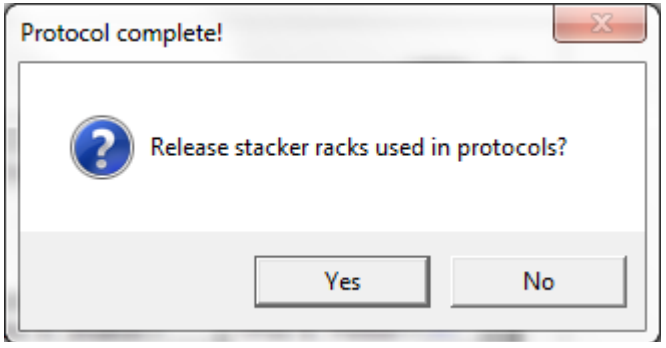
1 Verify that **Simulation is off** is displayed on the status indicator (accessible by clicking **View** > **Control Toolbar**).

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 7 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 8 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.

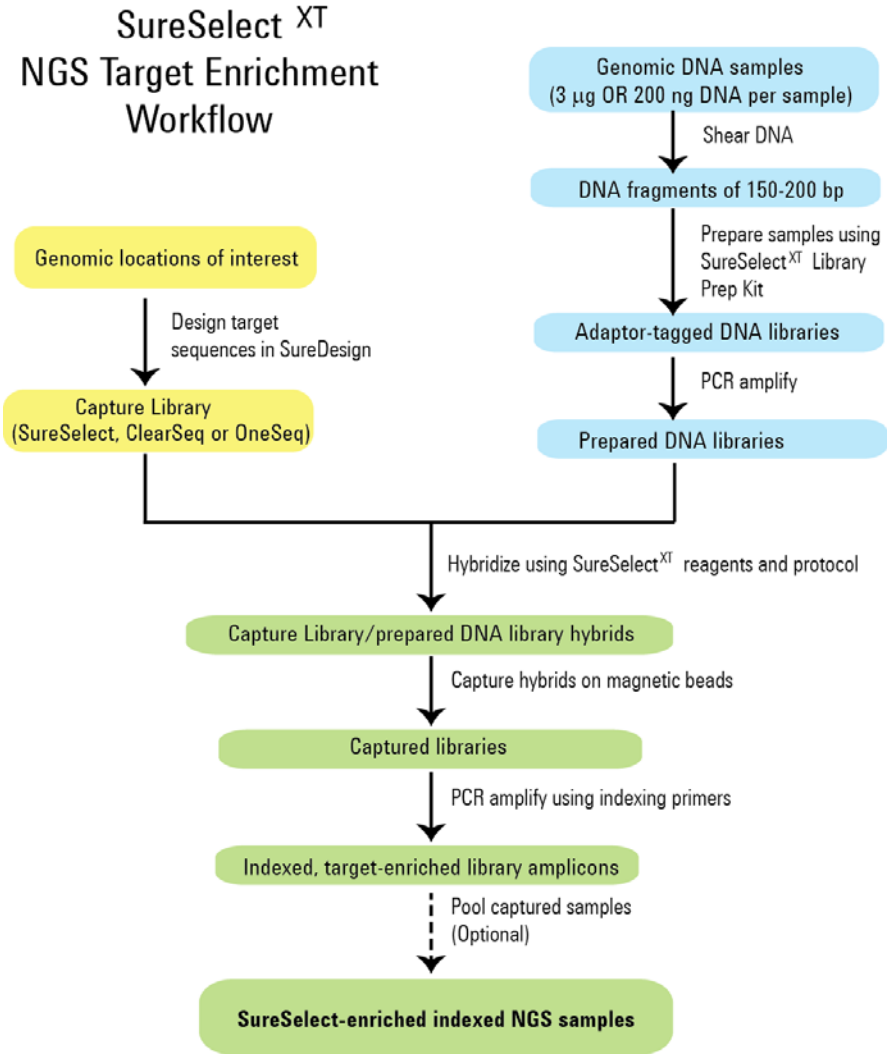


Figure 2 Overall sequencing sample preparation workflow.

Table 7 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 Capture Library	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 8 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 Capture Library	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 9 Columns to Samples Equivalency

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

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

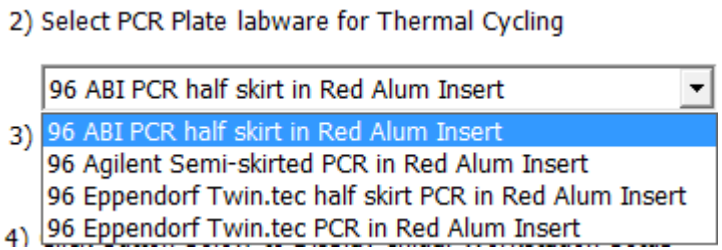
- The Agilent NGS Workstation processes samples column-wise beginning at column 1. gDNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample 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 Capture Library to each row of the plate. Plan your experiment such that each prepared DNA library corresponds to the appropriate Capture Library 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 Capture Libraries can require different amplification cycle numbers, based on sizes of the captured targets. It is most efficient to process similar-sized Capture Libraries on the same plate. See Table 72 on page 139 to determine which Capture Libraries may be amplified on the same plate.

Considerations for Equipment Setup

- Some workflow steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the Agilent NGS 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 10.



CAUTION

The plates listed in Table 10 are compatible with the Agilent NGS Bravo and associated VWorks automation protocols, designed to support use of various thermal cyclers.

Accordingly, some plates listed in Table 10 are not compatible with the recommended SureCycler 8800 Thermal Cycler. When using the SureCycler 8800 Thermal Cycler in the SureSelect automation workflow, use 96 Agilent semi-skirted PCR plates.

When using a different thermal cycler in the workflow, be sure to select a PCR plate that is compatible with your thermal cycler and that is listed in Table 10.

Table 10 Ordering information for supported PCR plates

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

3 Sample Preparation (3 µg DNA Samples)

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 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.

   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.

   e. *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.

   Refer to the Covaris instrument user guide for more details.

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

   Keep the cap on the tube.

NOTE When using a Covaris E-series instrument to prepare multiple gDNA samples in the same experiment, you can also use the 96 microTUBE plate (see Table 4 on page 15) for the DNA shearing step.

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

   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 10.

The target peak size is 150 to 200 bp.

Table 10 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 septa, 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 11, using the plate orientations shown in Figure 3.

Table 11 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

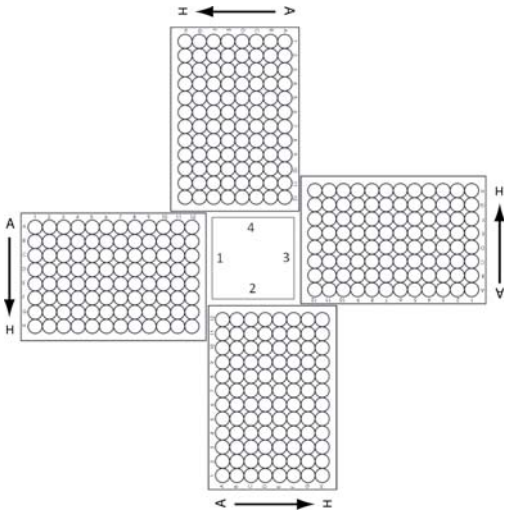


Figure 3 Agilent Labware MiniHub plate orientation. For Thermo Scientific reservoirs, place the notched corner facing the center of the hub.

10 Load the Bravo deck according to Table 12.

Table 12 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 13.

Table 13 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.

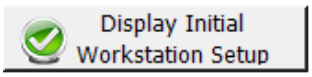
14 On the setup form, under **Select Protocol to Run**, select **AMPureXP_XT_ILM_v1.5.1.pro:Shearing-3 µg only.**

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.

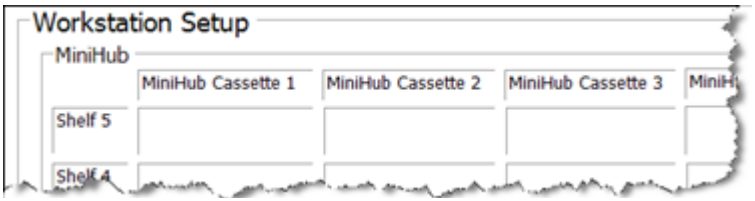
15 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate containing the sheared gDNA samples at position 9.

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

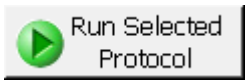
17 Click **Display Initial Workstation Setup**.



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 27 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. For more information, see the *Agilent DNA 1000 Kit Guide* at www.genomics.agilent.com.

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.

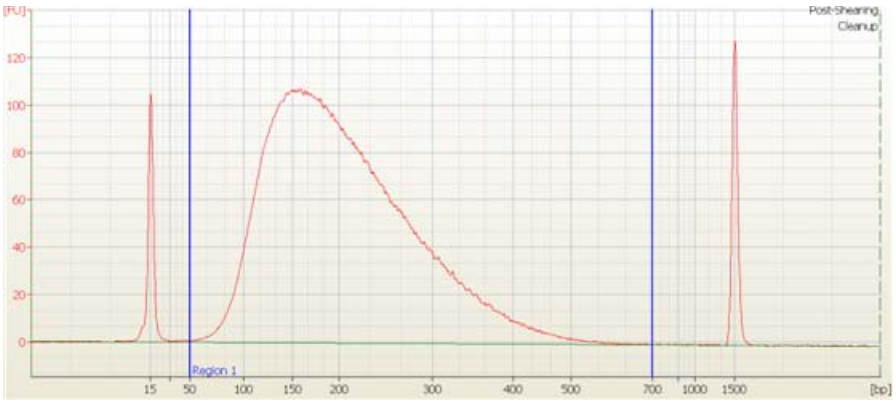


Figure 4 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

You can use Agilent's 4200 TapeStation or 2200 TapeStation for rapid analysis of multiple samples. Use a D1000 ScreenTape (p/n 5067-5582) and associated reagent kit (p/n 5067-5583). For more information to do this step, see the appropriate TapeStation User Manual at www.genomics.agilent.com.

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 instrument user manual. Use 1 µL of each sheared DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and D1000 sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

4 Load the sample plate or tube strips from step 3, the D1000 ScreenTape, and loading tips into the TapeStation as instructed in the instrument user manual. 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.

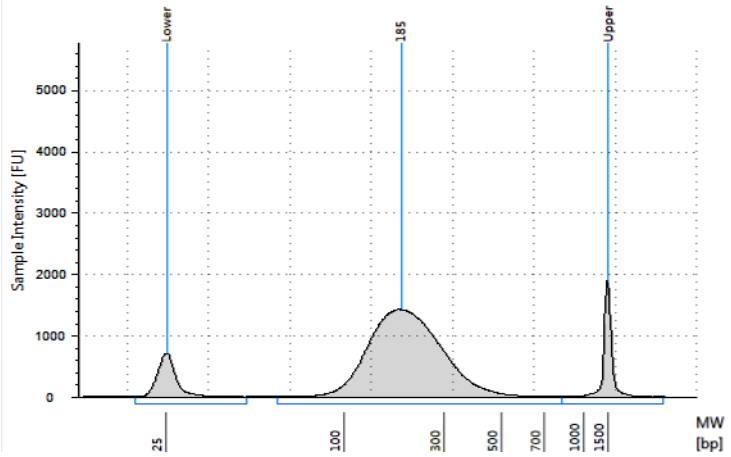


Figure 5 Analysis of sheared DNA using the 2200 TapeStation.

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 14. Mix well using a vortex mixer and keep on ice.

Table 14 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 15. Mix well using a vortex mixer and keep on ice.

Table 15 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 16. Mix well using a vortex mixer and keep on ice.

Table 16 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 17 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 17 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

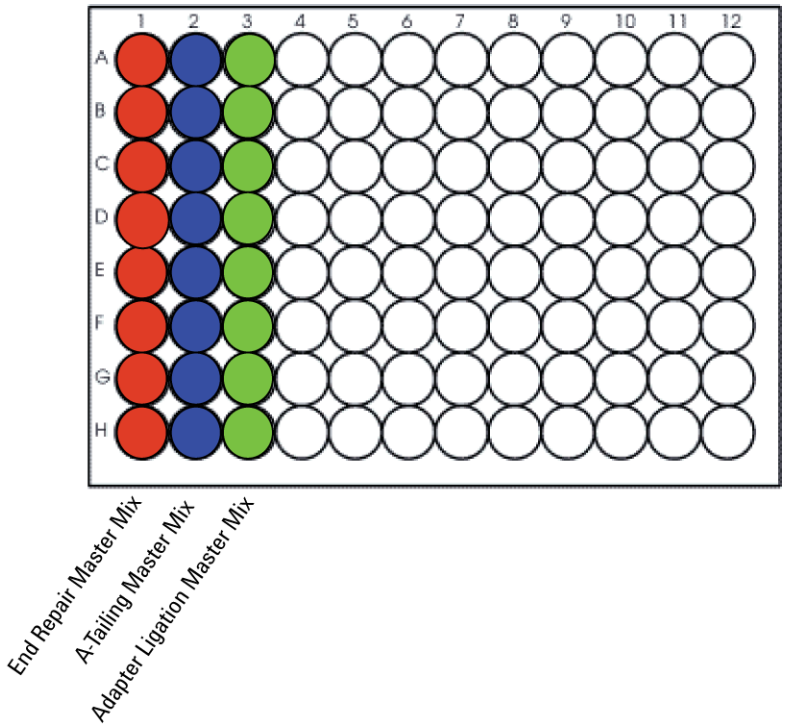


Figure 6 Configuration of the master mix source plate for LibraryPrep_XT_ILM_v1.5.1.rst

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 18, using the plate orientations shown in Figure 3.

Table 18 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 19.

Table 19 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 20.

Table 20 Initial BenchCel configuration for LibraryPrep_XT_ILM_v1.5.1.rst

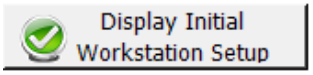
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	2 Tip boxes	Empty	Empty	Empty
2	4 Tip boxes	Empty	Empty	Empty
3	5 Tip boxes	Empty	Empty	Empty
4	7 Tip boxes	Empty	Empty	Empty
6	10 Tip boxes	Empty	Empty	Empty
12	11 Tip boxes	8 Tip boxes	Empty	Empty

Run VWorks runset LibraryPrep_XT_ILM_v1.5.1.rst

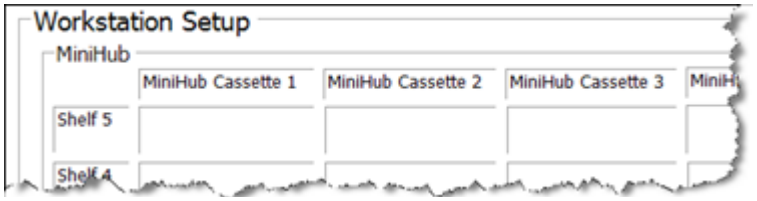
18 On the SureSelect setup form, under **Select Protocol to Run**, select **LibraryPrep_XT_ILM_v1.5.1.rst.**

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**.

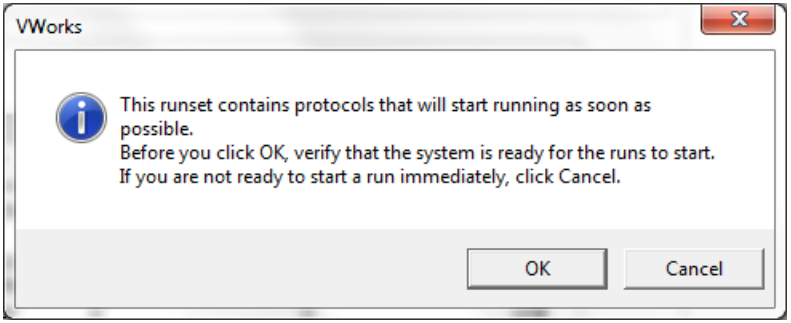


21 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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 21. Mix well using a vortex mixer and keep on ice.

Table 21 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 22 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 22 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.

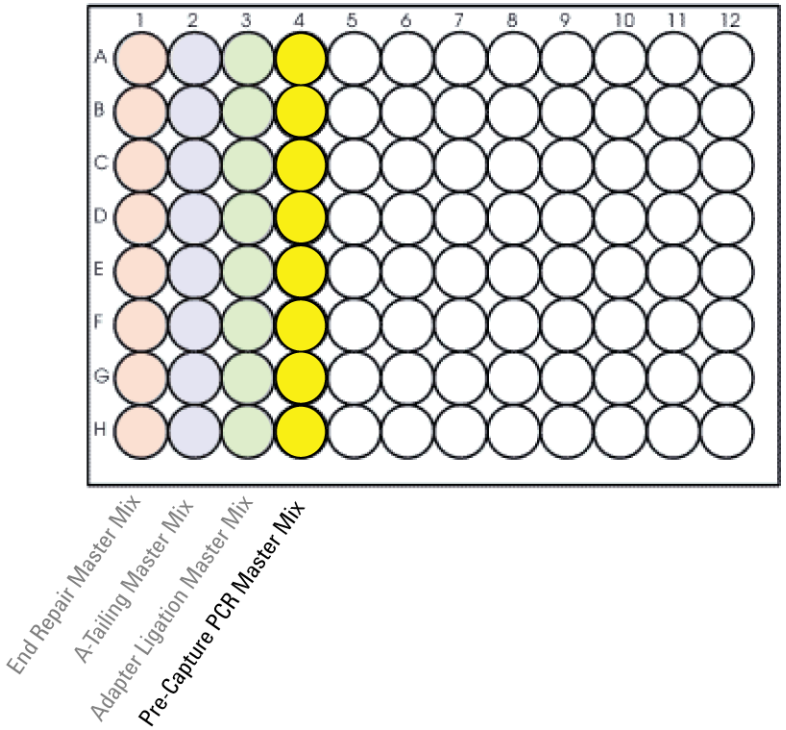


Figure 7 Configuration of the master mix source plate for Pre-CapturePCR_X-T_ILM_3µg_v1.5.1.pro. Columns 1-3 were used to dispense master mixes during the previous protocol.

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 23, using the plate orientations shown in Figure 3.

Table 23 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 24.

Table 24 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 25.

Table 25 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

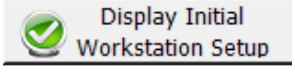
Run VWorks protocol Pre-CapturePCR_XT_ILM_3µg_v1.5.1.pro

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

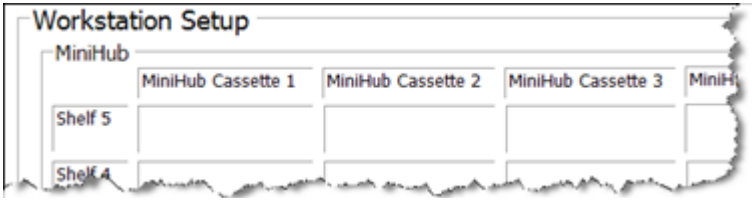
12 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

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

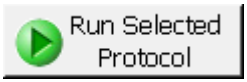
14 Click **Display Initial Workstation Setup**.



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

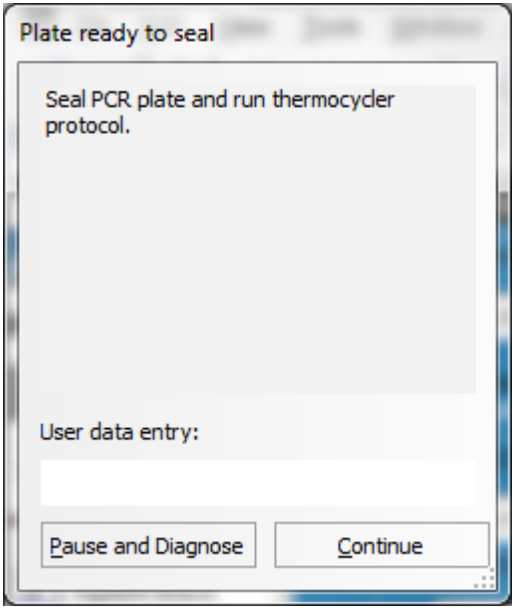


16 When verification is complete, click **Run Selected Protocol**.



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 26.

.

Table 26 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 27, using the plate orientations shown in Figure 3.

Table 27 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 28.

Table 28 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 29.

Table 29 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**.

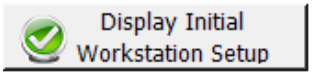
NOTE

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.

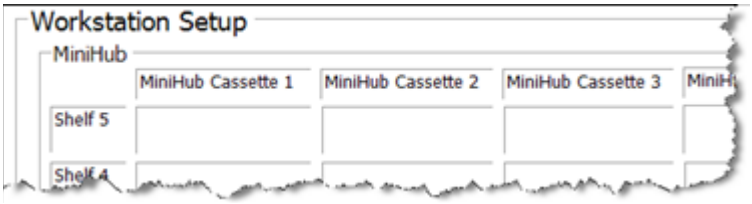
11 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate containing the amplified libraries at position 9.

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 to analyze the amplified libraries. For more information to do this step, see the *Agilent DNA 1000 Kit Guide* at www.genomics.agilent.com.

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.

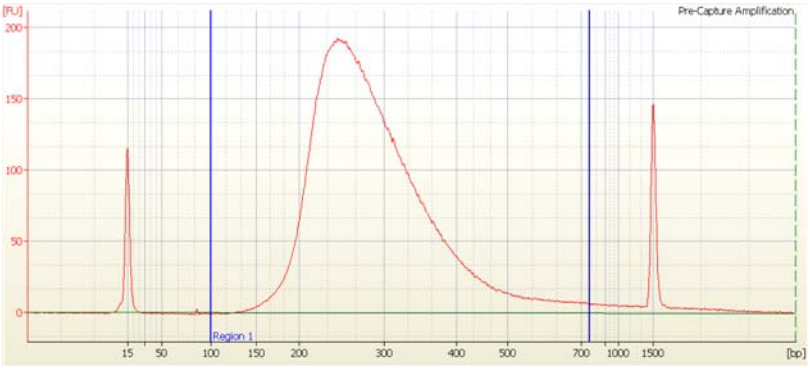


Figure 8 Analysis of amplified library DNA using a DNA 1000 assay.

Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape (p/n 5067-5582) and associated reagent kit (p/n 5067-5583) to analyze the amplified libraries. For more information to do this step, see the appropriate TapeStation User Manual at www.genomics.agilent.com.

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 instrument user manual. Use 1 µL of each amplified library DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and D1000 sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

4 Load the sample plate or tube strips from step 3, the D1000 ScreenTape, and loading tips into the TapeStation as instructed in the instrument user manual. 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.

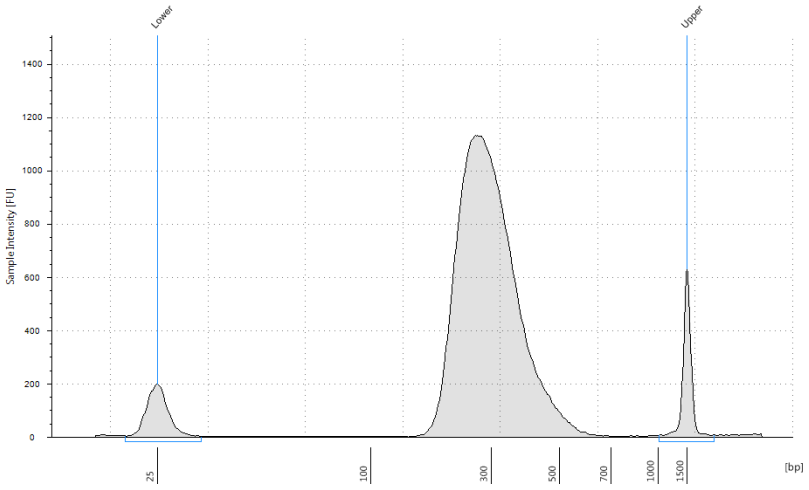


Figure 9 Analysis of amplified library DNA using the 2200 TapeStation.

4 Sample Preparation (200 ng DNA Samples)

This section contains instructions for the preparation of gDNA libraries from samples containing 200 ng of DNA. A separate protocol is provided on page 35 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 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.

   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.

   e *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.

   Refer to the Covaris instrument user guide for more details.

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

   Keep the cap on the tube.

NOTE When using a Covaris E-series instrument to prepare multiple gDNA samples in the same experiment, you can also use the 96 microTUBE plate (see Table 4 on page 15) for the DNA shearing step.

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

   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 30.

The target peak size is 150 to 200 bp.

Table 30 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 septa, 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 76.

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. See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information on doing this step.

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.

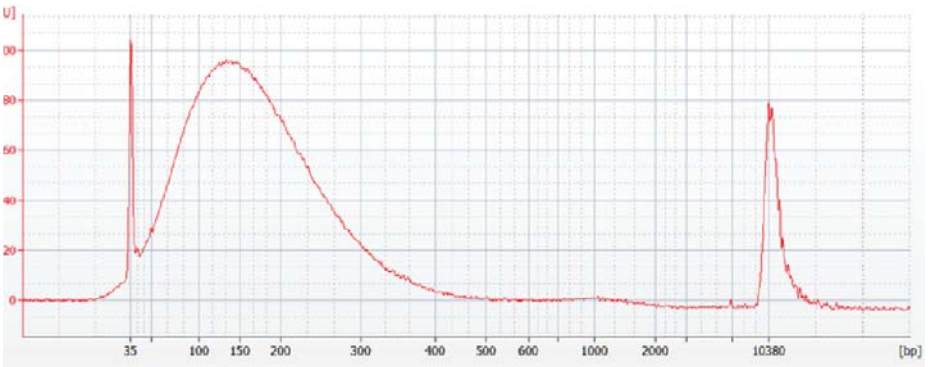


Figure 10 Analysis of sheared DNA using a High Sensitivity DNA Bioanalyzer assay.

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

Use a High Sensitivity D1000 ScreenTape (p/n 5067-5584) and reagent kit (p/n 5067-5585) to analyze the 200-ng sheared DNA samples. For more information to do this step, see the appropriate TapeStation User Manual at www.genomics.agilent.com.

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 instrument user manual. Use 2 µL of each indexed DNA sample diluted with 2 µL of High Sensitivity D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

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 instrument user manual. 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.

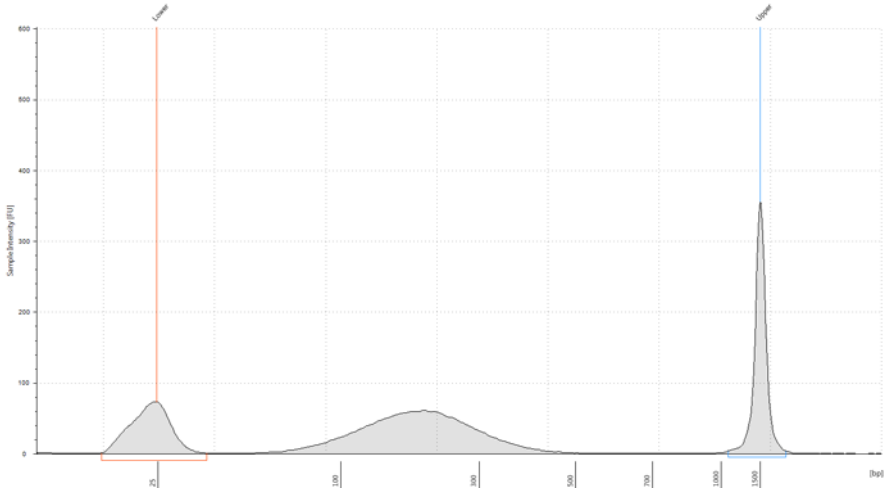


Figure 11 Analysis of sheared DNA using the 2200 TapeStation.

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 78 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 31. Mix well using a vortex mixer and keep on ice.

Table 31 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 32. Mix well using a vortex mixer and keep on ice.

Table 32 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 33. Mix well using a vortex mixer and keep on ice.

Table 33 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 34 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 34 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

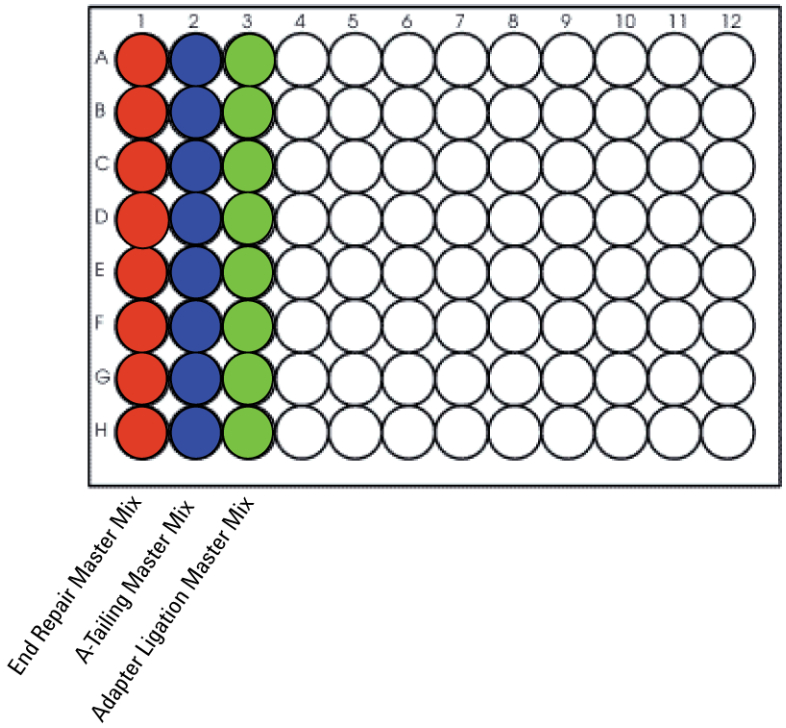


Figure 12 Configuration of the master mix source plate for LibraryPrep_XT_ILM_v1.5.1.rst

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 35, using the plate orientations shown in Figure 13.

Table 35 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

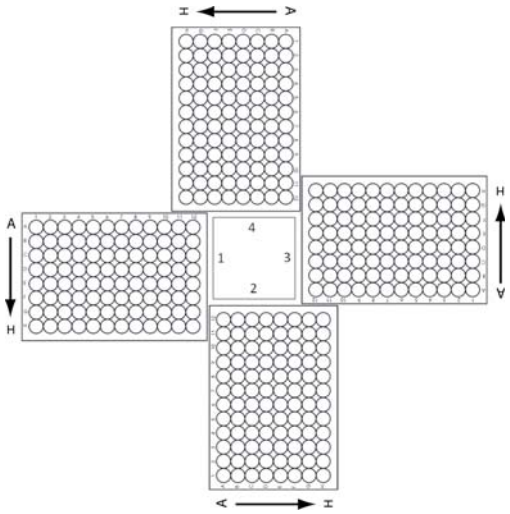


Figure 13 Agilent Labware MiniHub plate orientation. For Thermo Scientific reservoirs, place the notched corner facing the center of the hub.

16 Load the Bravo deck according to Table 36.

Table 36 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 37.

Table 37 Initial BenchCel configuration for LibraryPrep_XT_ILM_v1.5.1.rst

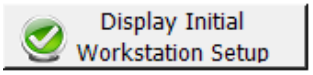
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	2 Tip boxes	Empty	Empty	Empty
2	4 Tip boxes	Empty	Empty	Empty
3	5 Tip boxes	Empty	Empty	Empty
4	7 Tip boxes	Empty	Empty	Empty
6	10 Tip boxes	Empty	Empty	Empty
12	11 Tip boxes	8 Tip boxes	Empty	Empty

Run VWorks runset LibraryPrep_XT_ILM_v1.5.1.rst

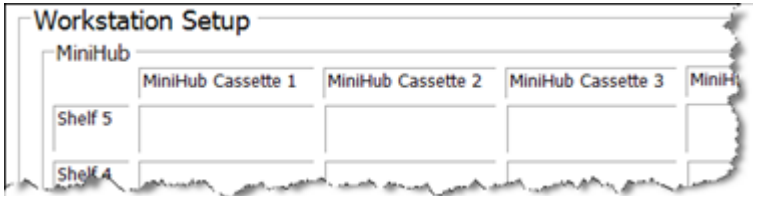
18 On the SureSelect setup form, under **Select Protocol to Run**, select **LibraryPrep_XT_ILM_v1.5.1.rst.**

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**.

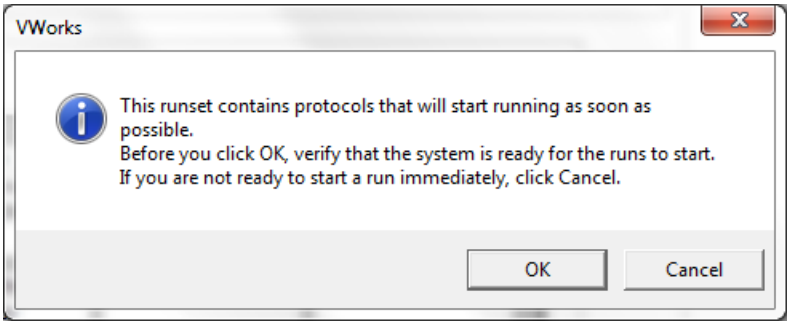


21 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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 38. Mix well using a vortex mixer and keep on ice.

Table 38 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 39 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 39 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.

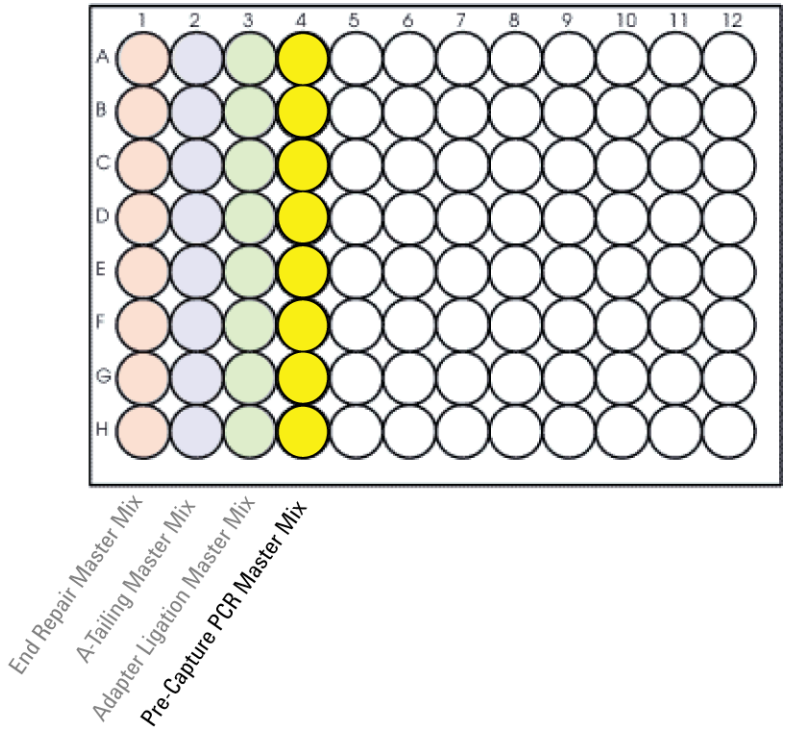


Figure 14 Configuration of the master mix source plate for Pre-CapturePCR_X-T_ILM_200ng_v1.5.1.pro. Columns 1-3 were used to dispense master mixes during the previous protocol.

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 40, using the plate orientations shown in Figure 13.

Table 40 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 41.

Table 41 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 42.

Table 42 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

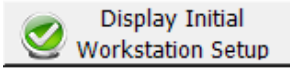
Run VWorks protocol Pre-CapturePCR_XT_ILM_200ng_v1.5.1.pro

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

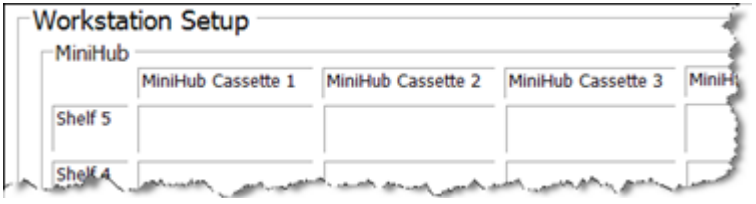
12 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

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**.



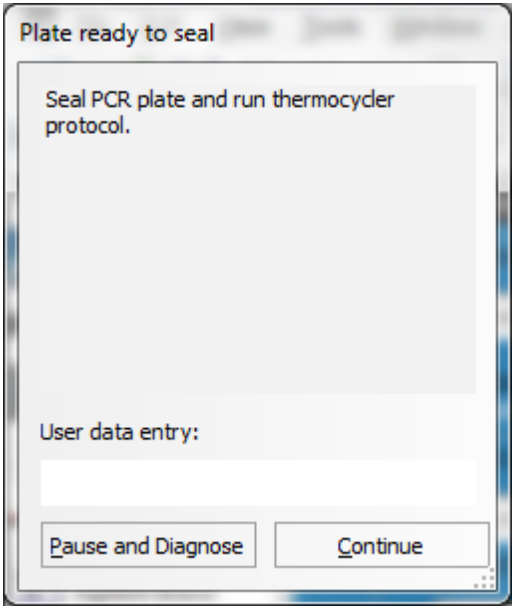
15 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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 43.

Table 43 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 44, using the plate orientations shown in Figure 13.

Table 44 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 45.

Table 45 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 46.

Table 46 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*

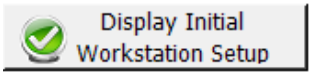
10 On the SureSelect setup form, under **Select Protocol to Run**, select **AMPureXP_XT_ILM_v1.5.1.pro:Pre- Capture PCR**.

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.

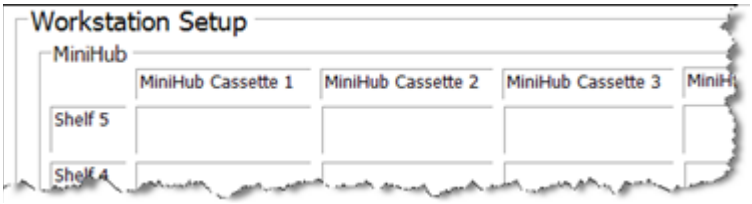
11 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate containing the amplified libraries at position 9.

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. For more information to do this step, see the *Agilent DNA 1000 Kit Guide* at www.genomics.agilent.com.

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.

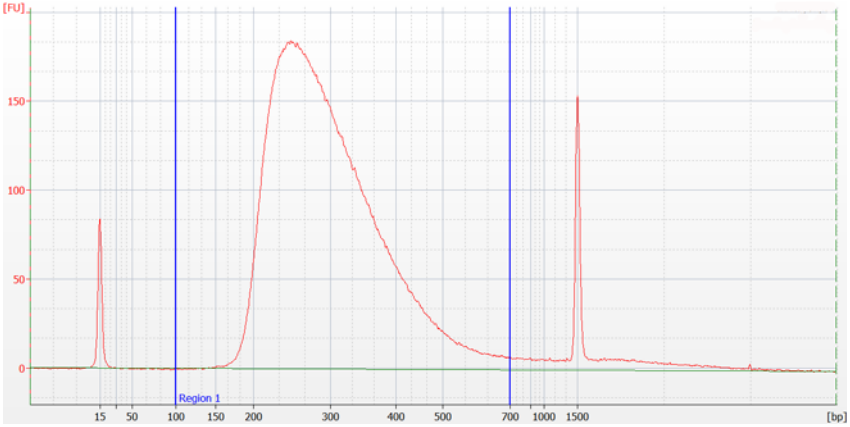


Figure 15 Analysis of amplified library DNA using a DNA 1000 assay.

Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape (p/n 5067-5582) and associated reagent kit (p/n 5067-5583) to analyze the amplified libraries. For more information to do this step, see the appropriate TapeStation User Manual at www.genomics.agilent.com.

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 instrument user manual. Use 1 µL of each amplified library DNA sample diluted with 3 µL of D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and D1000 sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

4 Load the sample plate or tube strips from step 3, the D1000 ScreenTape, and loading tips into the TapeStation as instructed in the instrument user manual. 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.

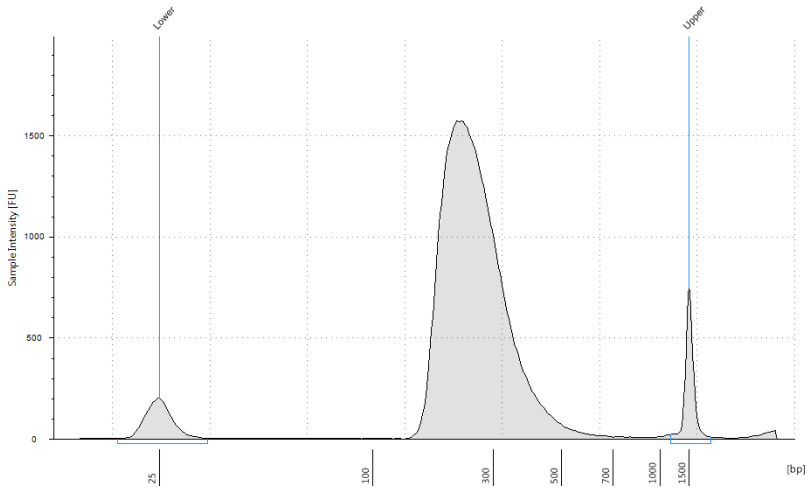


Figure 16 Analysis of amplified library DNA using the 2200 TapeStation.

5 Hybridization

This chapter describes the steps to combine the prepped library with the blocking agents and the SureSelect 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 SureSelect capture library 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.

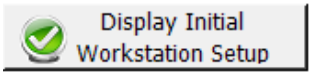
5 Load the Bravo deck according to Table 47.

Table 47 Initial Bravo deck configuration for Aliquot_Libraries_v1.5.1.pro

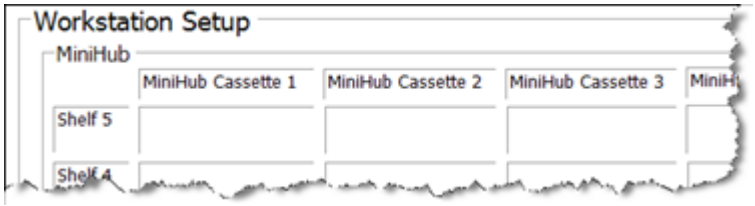
Location	Content
5	Empty Eppendorf plate
6	Empty tip box
8	New tip box
9	Prepped library DNA in Eppendorf plate

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

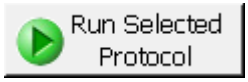
7 Click **Display Initial Workstation Setup**.



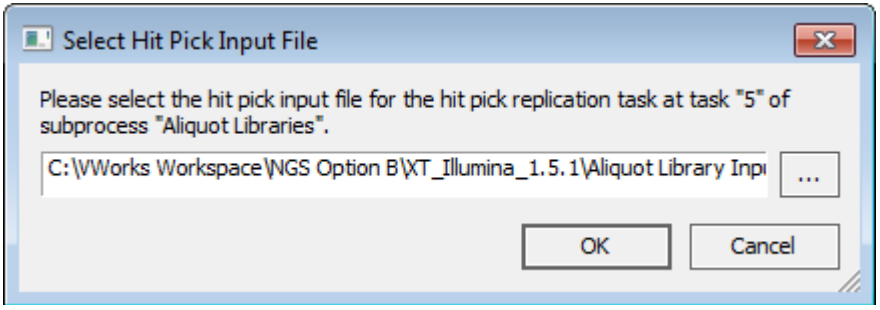
8 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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 Capture Library

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 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 48.

Table 48 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 SureSelect capture library master mix for each of the capture libraries that will be used for hybridization as indicated in Table 49 to Table 52. 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 Capture Library. However, capture libraries of different sizes require different post-capture amplification cycles. Plan experiments such that similar-sized libraries are hybridized on the same plate.

For runs that use a single capture library for all rows of the plate, prepare the master mix as described in Step a (Table 49 or Table 50) below.

For runs that use different capture libraries for individual rows, prepare each master mix as described in Step b (Table 51 or Table 52) below.

a **For runs that use a single capture library for all rows**, prepare the Capture Library Master Mix as listed in Table 49 or Table 50, based on the Mb target size of your design.

Table 49 Preparation of Capture Library Master Mix for target sizes <3.0 Mb, 8 rows of wells

Target size <3.0 Mb							
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	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
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 50 Preparation of Capture Library Master Mix for target sizes >3.0 Mb, 8 rows of wells

Target size >3.0 Mb							
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	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
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 capture libraries in individual rows**, prepare a Capture Library Master Mix for each capture library as listed in Table 51 or Table 52, based on the Mb target size of your design. The volumes listed in Table 51 and Table 52 are for a single row of sample wells. If a given capture library will be hybridized in multiple rows, multiply each of the values below by the number of rows assigned to that capture library.

Table 51 Preparation of Capture Library Master Mix for target sizes <3.0 Mb, single row of wells

Target size <3.0 Mb							
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	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
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 52 Preparation of Capture Library Master Mix for target sizes >3.0 Mb, single row of wells

Target size >3.0 Mb							
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	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
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 53.

Table 53 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 or bottle)	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 or bottle)	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 54 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 54 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

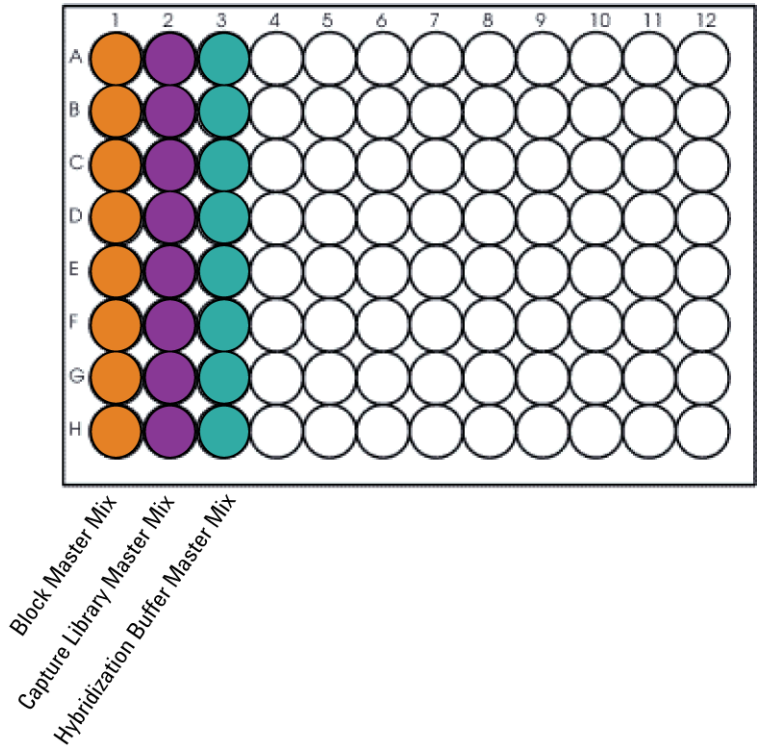


Figure 18 Configuration of the master mix source plate for Hybridization_v1.5.1.pro.

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 55, using the plate orientations shown in Figure 3.

Table 55 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 56.

Table 56 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 57.

Table 57 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

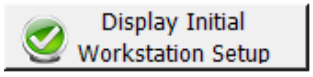
Run VWorks protocol Hybridization_v1.5.1.pro

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

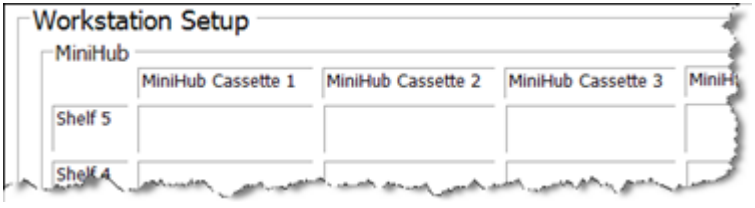
16 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 4 of the Bravo deck.

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

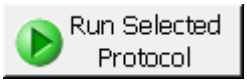
18 Click **Display Initial Workstation Setup**.



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

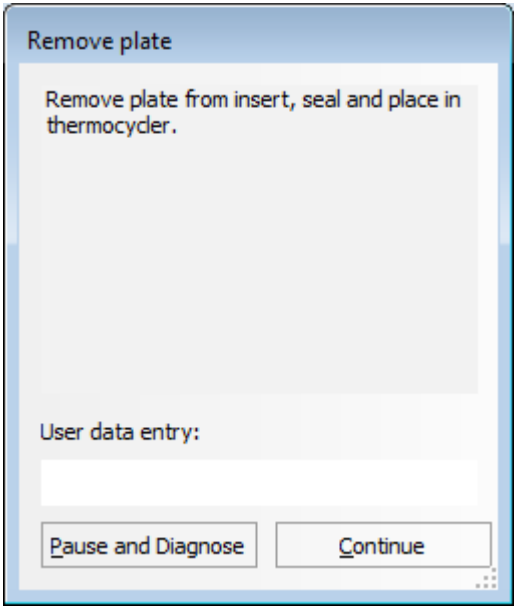


20 When verification is complete, click **Run Selected Protocol**.



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 58. After transferring the plate, click **Continue** on the VWorks screen.

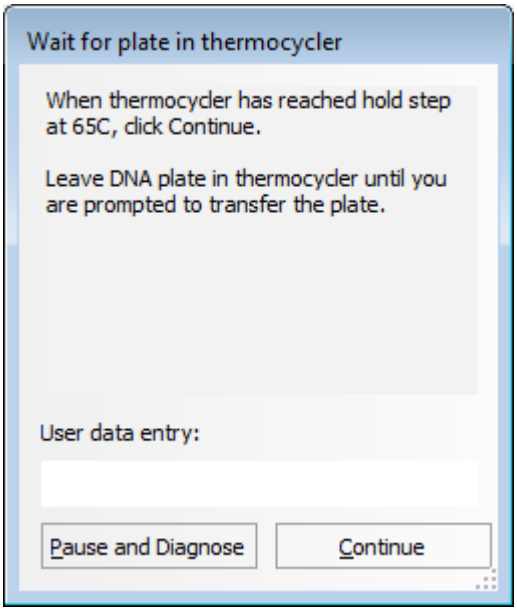
Table 58 Thermal cycler program used for sample denaturation prior to hybridization

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

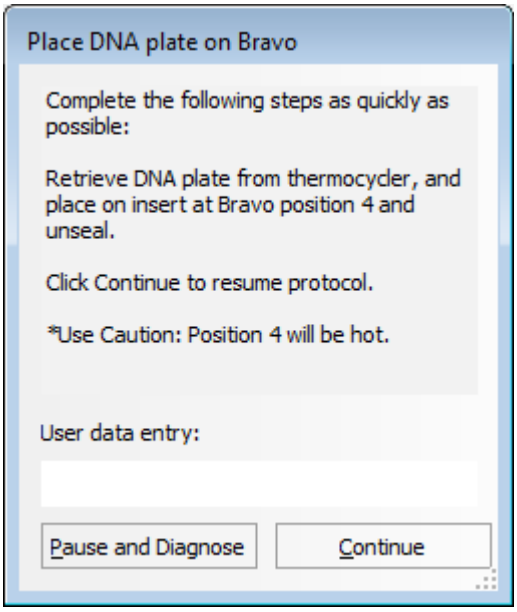
While the sample plate incubates on the thermal cycler, the Agilent NGS Workstation combines aliquots of the Capture Library Master Mix and Hybridization Buffer Master Mix.

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**.

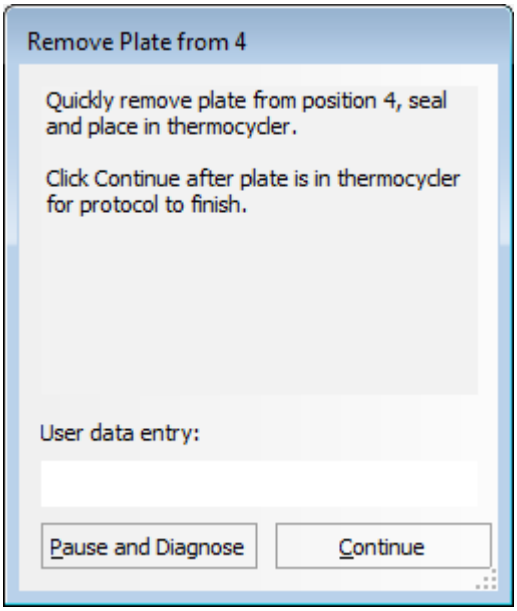


WARNING **Bravo deck position 4 will be hot.**

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 (see Table 4 on page 15 for ordering information).

Step 3. Capture the hybridized DNA

In this step, the gDNA-capture library 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 59

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 60. The volumes below include the required overage.

Table 60 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 61 below.

Table 61 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 62, using the plate orientations shown in Figure 3.

Table 62 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 63 (positions 5 and 6 should already be loaded).

Table 63 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 64.

Table 64 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

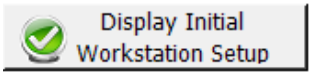
Run VWorks runset SureSelectCapture&Wash_v1.5.1.rst

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

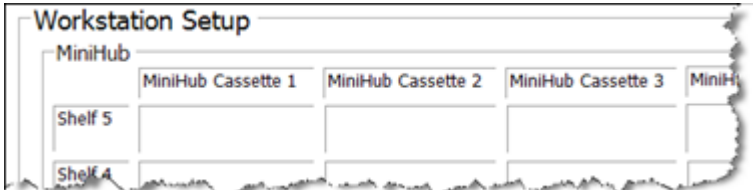
18 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used for hybridization. This plate will be transferred from the thermal cycler to Bravo deck position 4 when prompted by VWorks.

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

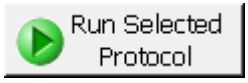
20 Click **Display Initial Workstation Setup**.



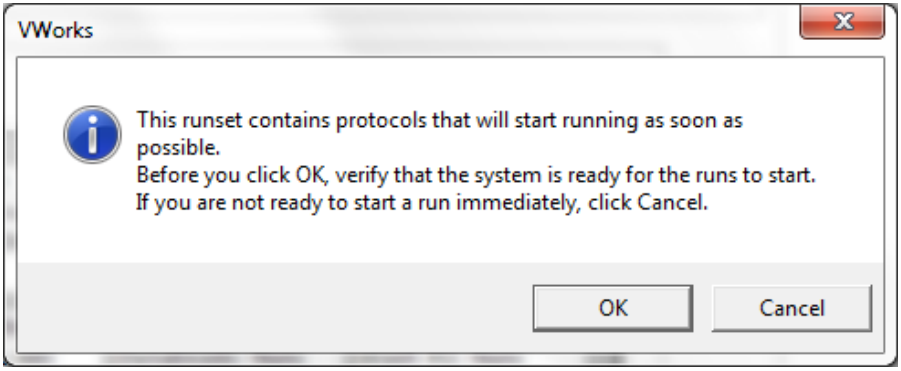
21 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

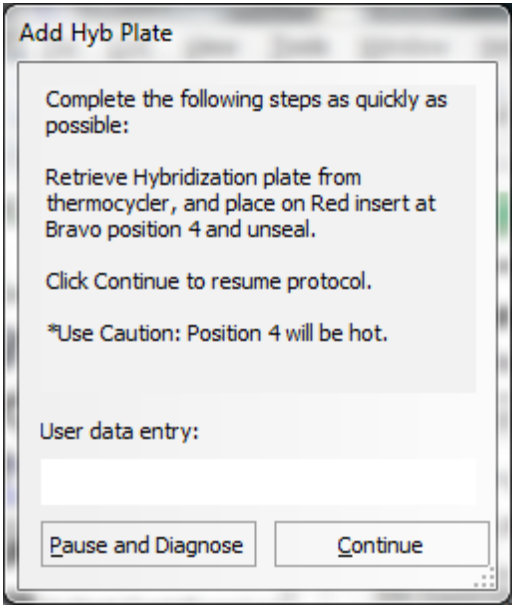


23 When ready to begin the run, click **OK** in the following window. If the temperature of Bravo deck position 4 was not pre-set to 66°C, the runset will pause while position 4 reaches temperature.



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.

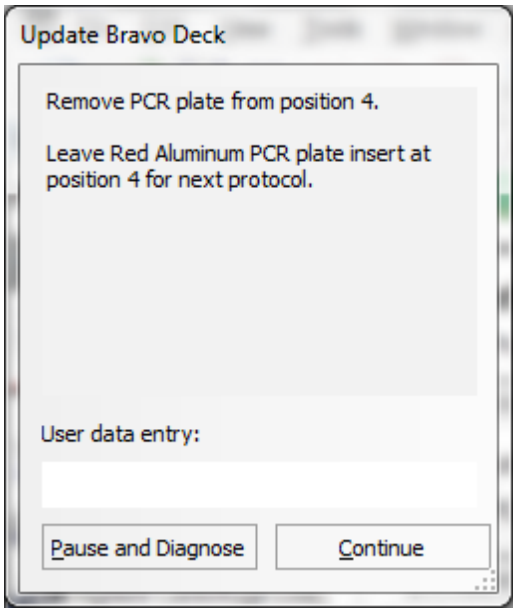
24 When prompted by VWorks as shown below, quickly remove the PCR plate, containing the hybridization reactions held at 65°C, from the thermal cycler. Unseal the plate carefully to avoid splashing, and quickly transfer the plate to position 4 of the Bravo deck, seated in the red insert. Click **Continue**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

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 size of your Capture Library determines the amplification cycle number used for indexing. Plan your experiments for amplification of samples prepared using Capture Libraries of similar sizes on the same plate. See Table 72 on page 139 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 Capture Library size.

As a guideline, Agilent recommends analyzing 100X amount of sequencing data compared to the Capture Library size for each sample. Specific examples of sequence data requirement recommendations are provided in Table 65. Calculate the number of indexes that can be combined per lane based on these guidelines.

Table 65 Sequencing data requirement guidelines

Capture Library Size	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 Capture Library 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 66. Mix well using a vortex mixer and keep on ice.

Table 66 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 67 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 67 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.

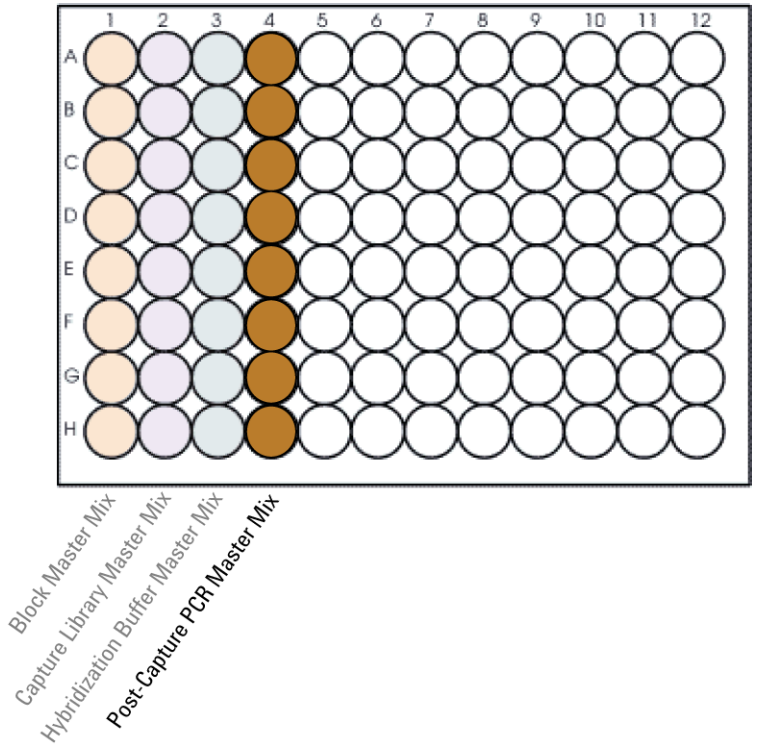


Figure 19 Configuration of the master mix source plate for Post-CaptureIndexing_XT_ILM_v1.5.1.pro. Columns 1–3 were used to dispense master mixes for the Hybridization_v1.5.1.pro protocol.

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 68, using the plate orientations shown in Figure 3.

Table 68 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 69.

Table 69 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 70.

Table 70 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

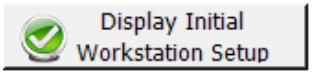
Run VWorks protocol Post-CaptureIndexing_XT_ILM_v1.5.1.pro

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

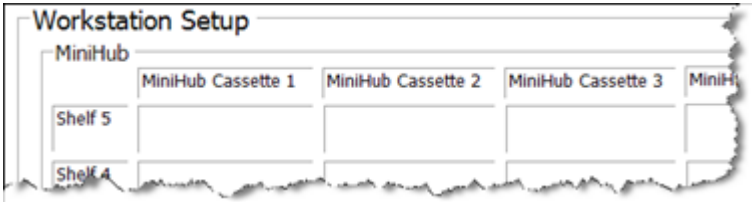
13 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

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**.



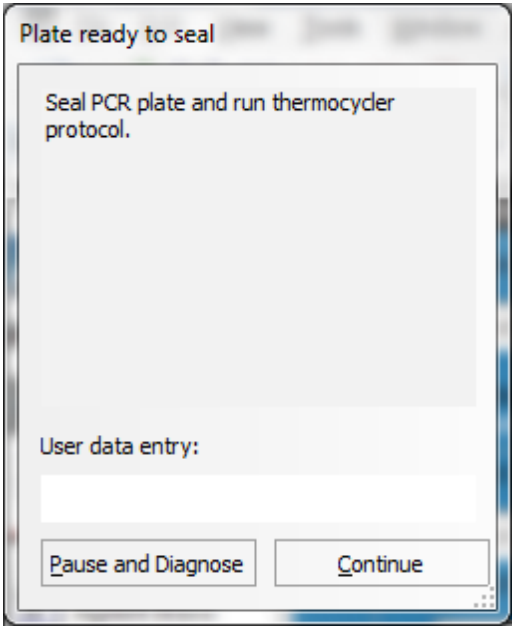
16 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

20 Transfer the PCR plate to a thermal cycler and run the PCR amplification program shown in Table 71 using the cycle number specified in Table 72.

Table 71 Post-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	10 to 16 Cycles see Table 72 for recommendations based on Capture Library 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 72 Recommended cycle number based on Capture Library size

Capture Library	Cycles
<0.5 Mb	16 cycles
0.5 to 1.49 Mb	14 cycles
> 1.5 Mb	12 cycles
All Exon and Exome libraries	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 Agencourt 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 73, using the plate orientations shown in Figure 3.

Table 73 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 74.

Table 74 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 75.

Table 75 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**.

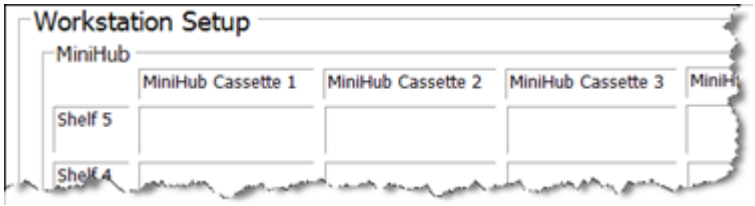
NOTE AMPureXP purification protocols are used during multiple steps of the SureSelect automation workflow. Be sure to select the correct workflow step when initiating the automation protocol.

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

1 Set up the 2100 Bioanalyzer as instructed in the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com.

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 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 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.

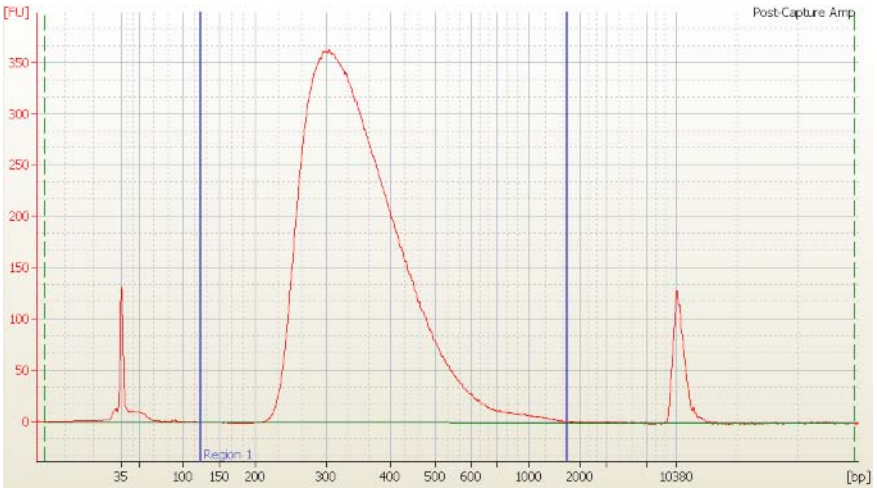


Figure 20 Analysis of indexed DNA using the High Sensitivity DNA Assay.

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

Use a High Sensitivity D1000 ScreenTape (p/n 5067-5584) and reagent kit (p/n 5067-5585) to analyze the indexed DNA. For more information to do this step, see the appropriate TapeStation User Manual at www.genomics.agilent.com.

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 instrument user manual. Use 2 µL of each indexed DNA sample diluted with 2 µL of High Sensitivity D1000 sample buffer for the analysis.

CAUTION Make sure that you thoroughly mix the combined DNA and High Sensitivity D1000 sample buffer on a vortex mixer for 5 seconds for accurate quantitation.

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 instrument user manual. 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.

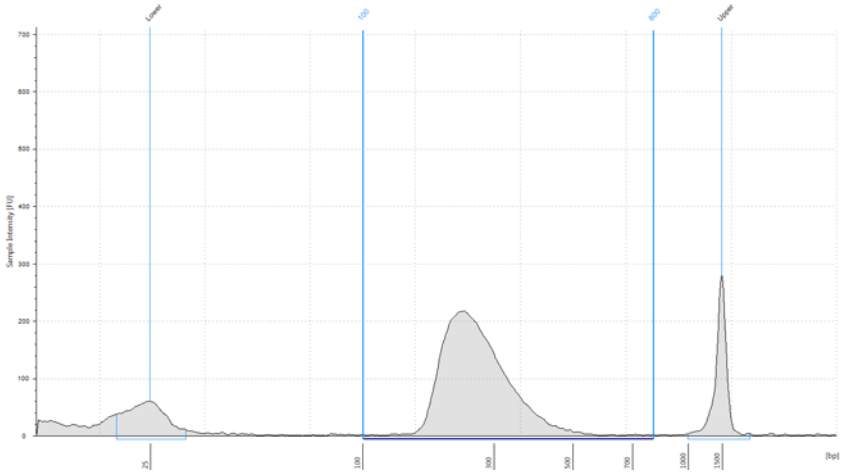


Figure 21 Analysis of indexed DNA using the 2200 TapeStation.

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 72 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 76 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 76 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 77 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 77 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.

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

Table 77 Illumina Kit Configuration Selection Guidelines

Platform	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
HiSeq 2500	Rapid Run	2 × 100 bp	200 Cycle Kit	v2	9–10 pM
HiSeq 2500	High Output	2 × 100 bp	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 × 76 bp	150 Cycle Kit	v3	12–16 pM
NextSeq 500/550	All Runs	2 × 100 bp	300 Cycle Kit	v2	1.5–1.8 pM
HiSeq 3000/4000	All Runs	2 × 100 bp	300 Cycle Kit	v1	180–190 pM

Sequencing run setup guidelines for 8-bp indexes

Sequencing runs must be set up to perform an 8-bp index read. See the Reference chapter for complete index sequence information.

For the HiSeq 2500 and NextSeq 500 (v1) platforms, use the *Cycles* settings shown in Table 78. Cycle number settings can be specified on the *Run Configuration* screen of the instrument control software interface after choosing *Custom* from the index type selection buttons.

Table 78 Cycle Number settings for HiSeq and NextSeq platforms

Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	9
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 79.

Table 79 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 85 on page 158).

7 Reference

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

Kit Contents

Each SureSelect^{XT} Automation Reagent Kit contains the following component kits:

Table 80 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

The contents of each of the component kits listed in Table 80 are described in the tables below.

Table 81 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 85 on page 158 for index sequences.

† See Table 84 on page 157 for a plate map.

Table 82 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 83 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 84 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 151 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 85 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 Illumina Paired-End Multiplexed Sequencing protocol using a SureSelect^{XT} Automated Reagent Kit and automation protocols provided with the Agilent NGS Workstation Option B.

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