SureSelect^{XT2} Automated Library Prep and Capture System

For Illumina Paired-End Multiplexed Sequencing

Automated using Agilent NGS Workstation Option B

Protocol

Version B1, June 2015

SureSelect platform manufactured with Agilent SurePrint Technology

For Research Use Only. Not for Use in Diagnostic Procedures.

Agilent Technologies

Notices

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Acknowledgement

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CAUTION

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In this Guide...

This guide describes an optimized protocol for Illumina paired-end multiplexed library preparation using the SureSelect^{XT2} 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 NGS Workstation.

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^{XT2} target enrichment protocol, and considerations for designing SureSelect^{XT2} experiments for automated processing using the Agilent NGS Workstation.

3 Sample Preparation

This chapter describes the steps to prepare index-tagged DNA samples for target enrichment.

4 Hybridization

This chapter describes the steps to pool indexed libraries and then hybridize and capture the pooled DNA.

5 Post-Capture Sample Processing for Multiplexed Sequencing

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

6 Reference

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

What's New in Version B.1

- Support for ClearSeq Capture Libraries, including ClearSeq Comprehensive Cancer Libraries (see Table 3 on page 13).
- Support for Human All Exon v6 Capture Libraries (see Table 2 on page 12).
- Update to name of end-repair reagent from *SureSelect End Repair Oligo Mix* to *SureSelect End Repair Nucleotide Mix* (see Table 65 on page 129).
- Update to name of indexing adaptor reagent from *SureSelect^{XT2} Pre-Capture Index* to *SureSelect Pre-Capture Indexed Adaptor* (see Table 65 on page 129).
- Updates to post-capture pooling and sequencing setup guidelines, including support for the NextSeq 500 platform (see page 121 to page 122).

What's New in Version B.0

- Support for kits supplied with either of two indexing primer configurations.

  Kits with revised index configuration (typically received February 2015 or later) include indexing primers A01 through H12 provided in a blue plate. For kit content details see page 128. For nucleotide sequences of the 8-bp indexes in this revised configuration, see Table 69 on page 131.

  Kits with original index configuration (typically received before February 2015), include indexing primers 1–96 provided in clear-capped tubes. For kit content details see page 132. For nucleotide sequences of the 8-bp indexes in this original configuration, see Table 75 on page 134 through Table 80 on page 139.
- Support for revised Library Prep kit configuration, now including End Repair Enzyme Mix and End Repair Oligo Mix (both replacing End Repair Master Mix). For instructions for use of the revised kit components, see page 44. See Table 65 on page 129 for updated kit contents.

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.

NOTE Agilent cannot guarantee the SureSelect Target Enrichment kits and cannot provide technical support for the use of non-Agilent protocols or instruments to process samples for enrichment.

Procedural Notes

- Certain protocol steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the Agilent NGS Workstation to allow rapid and efficient plate transfer.
- 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 6 on page 48.
- 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 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 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 vials used during an experiment on ice or in a cold block.
  4. If reagents will be used for multiple experiments, aliquot to multiple vials to minimize freeze/thaw cycles for each vial.
- 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 for SureSelect^{XT2} Automated Target Enrichment

Description	Vendor and part number
SureSelect or ClearSeq Capture Library*	Select one library from Table 2 or Table 3
SureSelect^{XT2} Automation Reagent Kit*†	Agilent
HiSeq platform (HSQ), 96 Samples	p/n G9661B
HiSeq platform (HSQ), 480 Samples	p/n G9661C
MiSeq platform (MSQ), 96 Samples	p/n G9662B
MiSeq platform (MSQ), 480 Samples	p/n G9662C
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Life Technologies p/n 4389764
Agencourt AMPure XP Kit	Beckman Coulter Genomics
60 mL	p/n A63881
450 mL	p/n A63882
Dynabeads MyOne Streptavidin T1	Life Technologies
2 mL	Cat #65601
10 mL	Cat #65602
100 mL	Cat #65603
Quant-iT dsDNA BR Assay Kit, for use with the Qubit fluorometer	
100 assays, 2-1000 ng	Life Technologies p/n Q32850
500 assays, 2-1000 ng	Life Technologies p/n Q32853
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023

* SureSelect reagents and Capture Libraries must be used within one year of receipt.

† HiSeq and MiSeq Reagent Kits are also compatible with the NextSeq 500 platform.

Table 2 SureSelect^{XT2} Automation Capture Libraries

Capture Library	96 Reactions	480 Reactions
SureSelect^{XT2} Human All Exon v6*	5190-8874	5 × 5190-8874
SureSelect^{XT2} Human All Exon v6 + UTRs*	5190-9306	5 × 5190-9306
SureSelect^{XT2} Human All Exon v6 + COSMIC*	5190-9312	5 × 5190-9312
SureSelect^{XT2} Human All Exon v6 Plus 1*	5190-8877	5 × 5190-8877
SureSelect^{XT2} Human All Exon v6 Plus 2*	5190-8880	5 × 5190-8880
SureSelect^{XT2} Clinical Research Exome*	5190-7347	5 × 5190-7347
SureSelect^{XT2} Focused Exome*	5190-7799	5 × 5190-7799
SureSelect^{XT2} Focused Exome Plus 1*	5190-7807	5 × 5190-7807
SureSelect^{XT2} Focused Exome Plus 2*	5190-7810	5 × 5190-7810
SureSelect^{XT2} Human All Exon v5*	5190-6218	5 × 5190-6218
SureSelect^{XT2} Human All Exon v5 + UTRs*	5190-6223	5 × 5190-6223
SureSelect^{XT2} Human All Exon v5 + lncRNA*	5190-6454	5 × 5190-6454
SureSelect^{XT2} Human All Exon v5 Plus*	5190-6225	5 × 5190-6225
SureSelect^{XT2} Human All Exon v4 *	5190-4668	5190-4670
SureSelect^{XT2} Human All Exon v4+ UTRs*	5190-4673	5190-4675
SureSelect^{XT2} Mouse All Exon*	5190-4683	5190-4685
SureSelect^{XT2} Custom 1 kb up to 499 kb†	5190-4848	5190-4850
(reorder)	(5190-4853)	(5190-4855)
SureSelect^{XT2} Custom 0.5 Mb up to 2.9 Mb†	5190-4858	5190-4860
(reorder)	(5190-4863)	(5190-4865)
SureSelect^{XT2} Custom 3 Mb up to 5.9 Mb†	5190-4868	5190-4870
(reorder)	(5190-4873)	(5190-4875)
SureSelect^{XT2} Custom 6 Mb up to 11.9 Mb†	5190-4878	5190-4880
(reorder)	(5190-4883)	(5190-4885)
SureSelect^{XT2} Custom 12 Mb up to 24 Mb†	5190-4888	5190-4890
(reorder)	(5190-4893)	(5190-4895)

* Eight gDNA samples are enriched in one capture reaction after sample pooling. Capture Libraries are provided for the number of capture reactions needed to enrich the indicated number of samples.

† Sixteen gDNA samples are enriched in one capture reaction after sample pooling. Capture Libraries are provided for the number of capture reactions needed to enrich the indicated number of samples.

Table 3 Compatible ClearSeq Automation Capture Libraries

Capture Library	96 Reactions	480 Reactions
ClearSeq Comprehensive Cancer XT2*	5190-8019	5 × 5190-8019
ClearSeq Comprehensive Cancer Plus XT2*	5190-8022	5 × 5190-8022
ClearSeq Inherited Disease XT2†	5190-7526	5 × 5190-7526
ClearSeq Inherited Disease Plus XT2†	5190-7529	5 × 5190-7529
ClearSeq DNA Kinome XT2*	5190-4678	5190-4680

* Sixteen gDNA samples are enriched in one capture reaction after sample pooling. Capture Libraries are provided for the number of capture reactions needed to enrich the indicated number of samples.

† Eight gDNA samples are enriched in one capture reaction after sample pooling. Capture Libraries are provided for the number of capture reactions needed to enrich the indicated number of samples.

Required Equipment

Table 4 Required Equipment for SureSelect^{XT2} Automated Target Enrichment

Description	Vendor and part number
Agilent NGS Workstation Option B, with VWorks software version 11.3.0.1195	Contact Agilent Automation Solutions for ordering information: Customerservice.automation@agilent.com
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
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, 2.2 mL, Square Well (waste reservoirs)	Axygen p/n P-2ML-SQ-C E & K Scientific p/n EK-2440
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 See page 36 for a list of supported PCR plates for automation protocols	Agilent p/n 401334
Covaris Sample Preparation System, E-series or S-series	Covaris
Covaris sample holders	
96 microTUBE plate (E-series only)	Covaris p/n 520078
microTUBE for individual sample processing	Covaris p/n 520045
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Qubit Fluorometer	Life Technologies p/n Q32857
Qubit assay tubes	Life Technologies p/n Q32856

Table 4 Required Equipment for SureSelect^{XT2} Automated Target Enrichment

Description	Vendor and part number
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Magnetic separator	DynaMag-50 magnet, Life Technologies p/n 123-02D or equivalent
DNA Analysis Platform and Consumables	
2100 Bioanalyzer Laptop Bundle	Agilent p/n G2943CA
2100 Bioanalyzer Electrophoresis Set	Agilent p/n G2947CA
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
OR	
2200 TapeStation	Agilent p/n G2964AA or G2965AA
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
D1000 Reagents	Agilent p/n 5067-5585
NucleoClean Decontamination Wipes	Millipore p/n 3097
Vacuum concentrator	Savant SpeedVac, model DNA120, with 96-well plate rotor, model RD2MP, or equivalent
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Vortex mixer	

Optional Reagents

Table 5 Reagents for Optional Quantitation Methods

Description	Vendor and part number
QPCR NGS Library Quantification Kit (Illumina)	Agilent p/n G4880A

Optional Equipment

Table 6 Equipment for Optional Quantitation Methods

Description	Vendor and part number
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

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^{XT2} target enrichment protocol, and considerations for designing SureSelect experiments for automated processing using the Agilent NGS Workstation.

About the Agilent NGS Workstation

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.

CAUTION Before you begin, make sure that you have read and understand operating, maintenance and safety instructions for using your Bravo platform. Refer to the *Bravo Platform User Guide* (G5409-90006) and the *VWorks Software User Guide* (G5415-90063).

Bravo Platform Deck

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

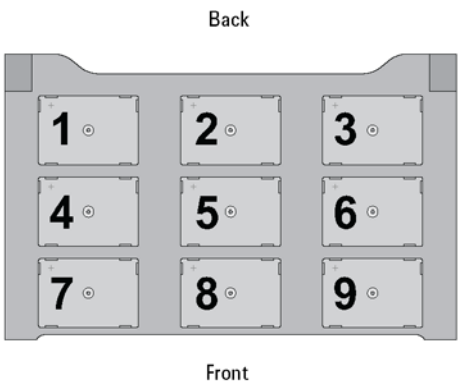


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

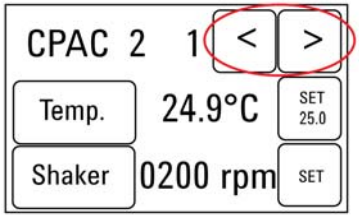
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

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

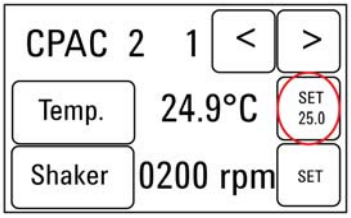
Table 7 Inheco Multi TEC Control touchscreen designations

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

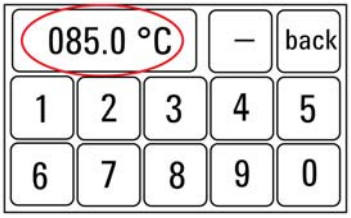
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



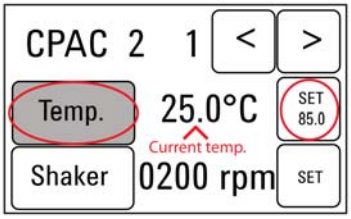
2 To set the temperature of the selected block, press the SET button.



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



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



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

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

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

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

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

3 Press the **START** button.

The ThermoCube will then initates 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 11.3.0.1195.

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

Logging in to the VWorks software

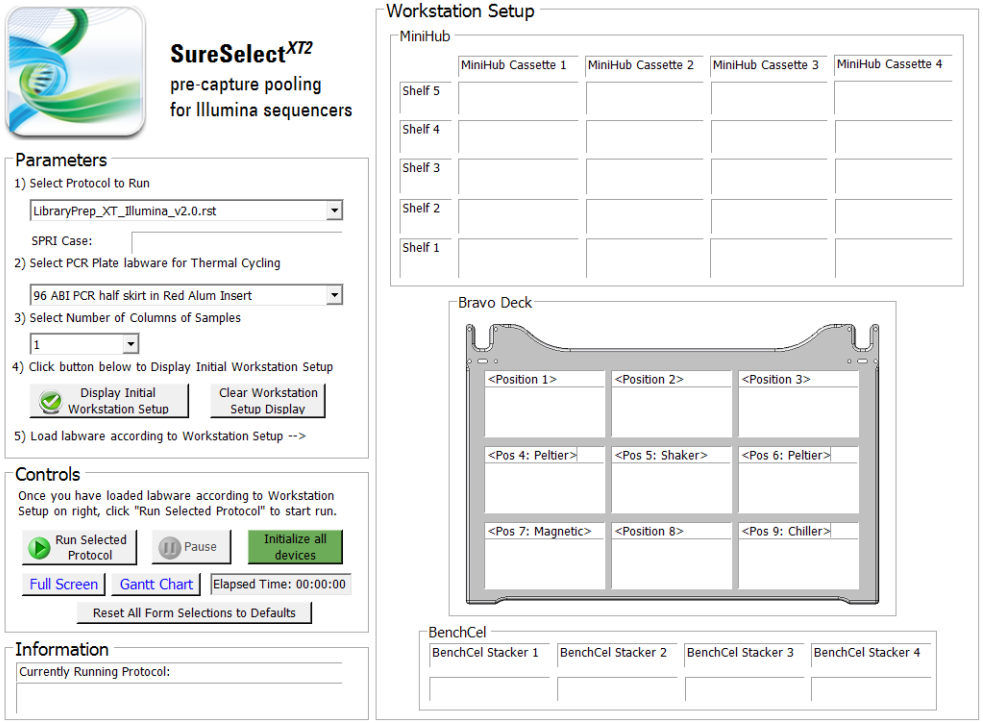
1. Double-click the XT2_ILM.VWForm shortcut on the Windows desktop to start the VWorks software.
2. If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
3. In the User Authentication dialog, type your VWorks user name and password, and click **OK**. (If no user 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.

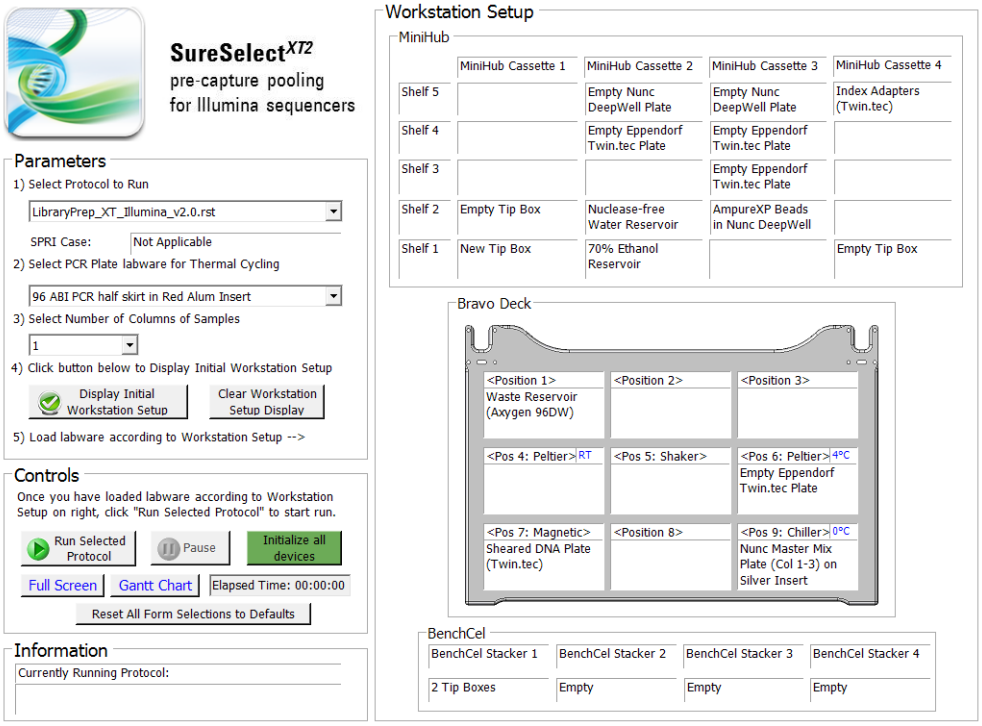
Using the XT2_ILM.VWForm.VWForm to setup and start a run

Use the VWorks form XT2_ILM.VWForm.VWForm, shown below, to set up and start each SureSelect automation protocol or runset.



1. Open the form using the XT2_ILM.VWForm.VWForm shortcut on your desktop.
2. Use the drop-down menus on the form to select the appropriate SureSelect^{XT2} workflow step, PCR plate labware description, 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.

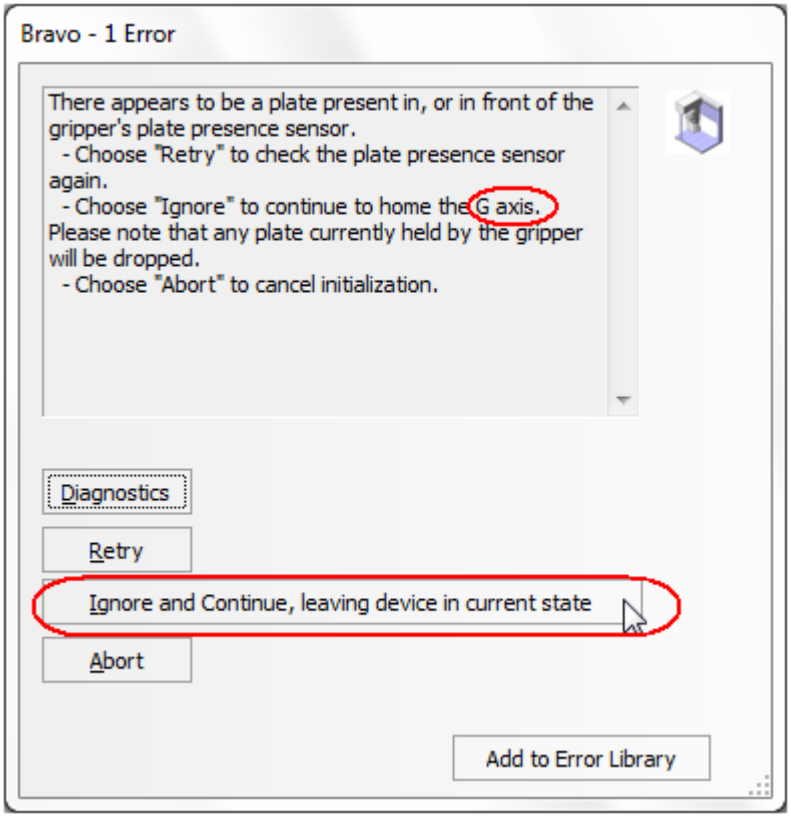


5 After verifying that the NGS Workstation has been set up correctly, click **Run Selected Protocol**.

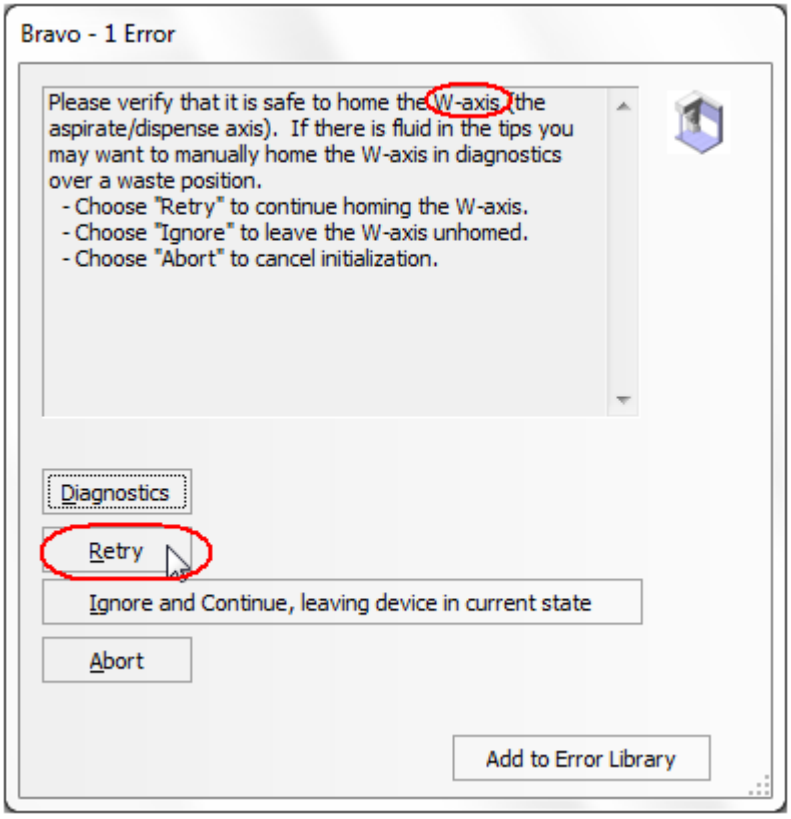
Error messages encountered at start of run

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**.



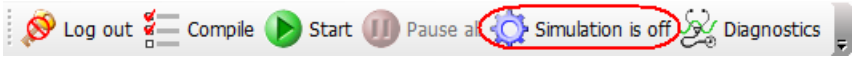
2 If you encounter the W-axis error message shown below, select **Retry.**



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**).

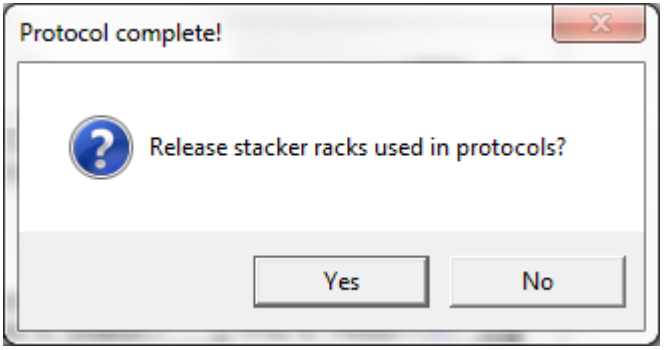


2 If the indicator displays **Simulation is on,** click the status indicator button to turn off the simulation mode.

NOTE If you cannot see the toolbar above the VWorks form, click **Full Screen on/off** 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 Workflow

Figure 2 summarizes the SureSelect^{XT2} pre-capture indexing and target enrichment workflow. For each sample to be sequenced, an individual library indexing reaction is performed. Indexed libraries are then pooled for hybridization and capture steps, using a pooling strategy appropriate for the size of the Capture Library and the sequencing design.

Table 8 summarizes how the VWorks protocols are integrated into the SureSelect^{XT2} workflow. See the Sample Preparation, Hybridization, and Post-Capture Sample Processing for Multiplexed Sequencing chapters for complete instructions for use of the VWorks protocols for sample processing.

For greater flexibility, two versions of the Hybridization automation protocol are available (see Table 8). The Hybridization_MMCol_v2.0.pro protocol is used optimally when processing full plates of hybridization samples and may be set up using different Capture Libraries in each row, allowing enrichment with up to 8 different libraries in a run. The Hybridization_MMRow_v2.0.pro protocol is designed for optimal reagent usage when processing plates containing ≤6 columns of samples and may be set up using different Capture Libraries in each column, allowing enrichment with up to 12 different libraries in a run.

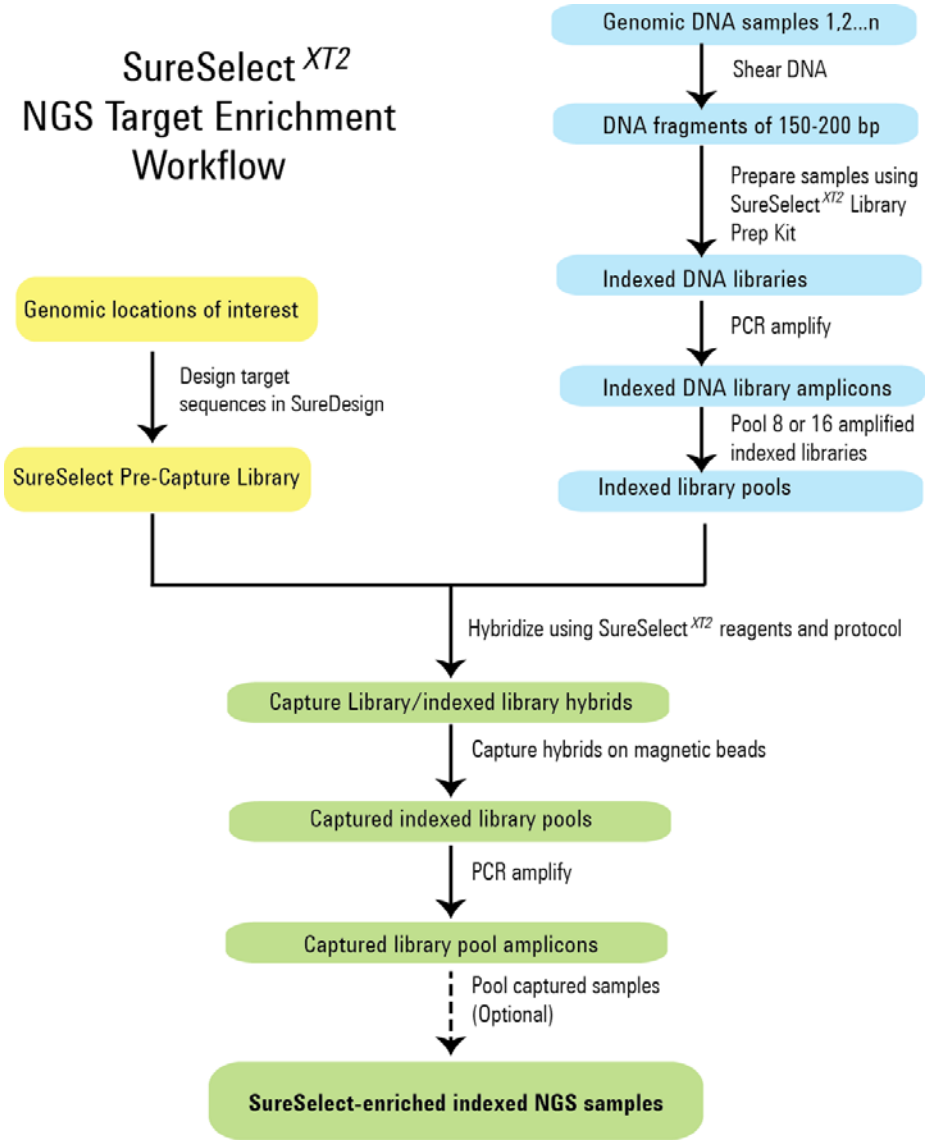


Figure 2 Overall sequencing sample preparation workflow.

Table 8 Overview of VWorks protocols and runsets used during the workflow

Workflow Step (Protocol Chapter)	Substep	VWorks Protocols Used for Agilent NGS Workstation automation
Sample Preparation	Prepare indexing adaptor-ligated DNA	LibraryPrep_XT_Illumina_v2.0.rst
	Amplify indexed DNA	Pre-CapturePCR_XT_Illumina_v2.0.pro
	Purify indexed DNA amplicons using AMPure XP beads	SPRI_XT_Illumina_v2.0.pro:Pre-Capture PCR Cleanup
Hybridization	Prepare indexed DNA pools for hybridization	PreCapture_Pooling_v1.0.pro (initiated using XT2_Pooling.VWForm)
	Hybridize pooled indexed DNA to Capture Library	Hybridization_MMCol_v2.0.pro OR Hybridization_MMRow_v2.0.pro
	Capture and wash DNA hybrids	SureSelectCapture&Wash_v2.0.rst
Sample Processing for Multiplexed Sequencing	PCR amplify captured DNA	Post-CaptureOnBeadPCR_XT_Illumina_v2.0.pro
	Purify captured DNA amplicons using AMPure XP beads	SPRI_XT_Illumina_v2.0.pro:Post-CaptureOnBeadPCR Cleanup

Experimental Setup Considerations for Automated Runs

SureSelect^{XT2} automated Library Prep runs may include 1, 2, 3, 4, 6, or 12 columns (equivalent to 8, 16, 24, 32, 48, or 96 wells) of gDNA samples. Plan your experiments using complete columns of samples.

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 Library Prep reactions configured as 4 runs of 3 columns of samples per run.

Prior to hybridization, indexed library samples are pooled in sets of 8 samples or in sets of 16 samples, based on the type of Capture Library to be used for hybridization (see Table 27 on page 65). Thus one Library Prep run corresponds to 0.5 to 12 hybridization wells, depending on the number of columns processed (see Table 9). Hybridization runs are set up using 1, 2, 3, 4, 6, or 12 columns (equivalent to 8, 16, 24, 32, 48, or 96 wells). Accordingly, it is typically beneficial to consolidate the indexed library pools from multiple Library Prep runs to prepare full columns of samples for Hybridization runs and downstream workflow steps.

Table 9 Hybridization reaction numbers derived from each Library Prep run size

Number of Columns Processed in Library Prep Protocol	Total Libraries Prepared	Number of Hyb Reactions	
		Pools containing 8 indexed libraries	Pools containing 16 indexed libraries
1	8	1	0.5
2	16	2	1
3	24	3	1.5
4	32	4	2
6	48	6	3
12	96	12	6

Optimal reagent usage is obtained using Hybridization runs that include 3, 6, or 12 columns. Hybridization runs of this size result from processing indexed library samples from multiple 96-well plates in the same Hybridization run. To determine the number of Library Prep reaction plates required for various Hybridization run sizes, see Table 10 for All Exon captures and see Table 11 for all other captures. Sample numbers required for optimal 3, 6, and 12 column runs are highlighted in gray.

For greatest efficiency of reagent use, plan experiments using at least 3 columns per run. Each 2x96-reaction kit contains sufficient reagents for hybridization reactions configured as 1 run of 3 columns of samples per run.

Table 10 Sample number conversion using Exome libraries (8-sample pools)

Number of 96-Well Plates Processed though Library Prep	Total Indexed Libraries Prepared	Number of Hybridization Reactions	Columns of Samples in Hybridization Protocol
1	96	12	1.5*
2	192	24	3
3	288	36	4.5*
4	384	48	6
5	480	60	7.5*
6	576	72	9†
7	672	84	10.5*
8	768	96	12

* Not a valid run size. Hybridization runs should include 1, 2, 3, 4, 6, or 12 complete columns of samples.

† When planning a run using 6 plates of gDNA samples to generate 9 columns of Hybridization samples, split the Hybridization samples into one 6-column plate and one 3-column plate.

Table 11 Sample number conversion using 16-sample pools

Number of 96-Well Plates Processed though Library Prep	Total Indexed Libraries Prepared	Number of Hybridization Reactions	Columns of Samples in Hybridization Protocol
1	96	6	0.75*
2	192	12	1.5*
3	288	18	2.25*
4	384	24	3
5	480	30	3.75*
6	576	36	4.5*
7	672	42	5.25*
8	768	48	6
9	864	54	6.75*
10	960	60	7.5*
11	1056	66	8.25*
12	1152	72	9†
13	1248	78	9.75*
14	1344	84	10.5*
15	1440	90	11.25*
16	1536	96	12

* Not a valid run size. Hybridization runs should include 1, 2, 3, 4, 6, or 12 complete columns of samples.

† When planning a run using 12 plates of gDNA samples to generate 9 columns of Hybridization samples, split the Hybridization samples into one 6-column plate and one 3-column plate.

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

- The Agilent NGS Workstation processes samples column-wise beginning at column 1. gDNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- Samples are indexed during the LibraryPrep_XT_Illumina_v2.0.rst runset using indexing adaptors supplied in the corresponding well on a separate plate. Assign the gDNA sample wells to be indexed with their respective indexing primers during experimental design.

Considerations for Indexed DNA Sample Placement for Automated Hybridization and Post-Hybridization Processing

Indexed DNA samples are pooled before the hybridization step (see Figure 2) and captured DNA samples may be pooled again when preparing samples for sequencing. It is important to develop a pooling strategy that is compatible with the specific Capture Library sizes and sequencing goals of the experiment using the following considerations:

- At the hybridization step (see Figure 2), you can add a different SureSelect or ClearSeq Capture Library to different rows or columns of the plate. See page 28 for guidelines on selecting the appropriate hybridization run configuration. Plan your experiment such that each indexed DNA library is placed in a pool in the row or column of the sample plate that corresponds to the appropriate Capture Library for hybridization.
- 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 57 on page 112 to determine which Capture Libraries may be amplified on the same plate.

- After the SureSelect^{XT2} capture process, DNA samples enriched using small Capture Libraries are typically pooled a second time before sequencing. See page 121 for post-capture secondary sample pooling guidelines. When using such a secondary pooling strategy, develop a pre-capture indexed library pooling strategy that is compatible with post-capture pooling and sequencing designs.

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, then centrifuged to collect any dispersed liquid, before being transfered between instruments. To maximize efficiency, locate the PlateLoc thermal microplate sealer and the centrifuge in close proximity to the Agilent NGS Workstation and thermal cycler.

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 SureSelect_RNA_ILM.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 12.

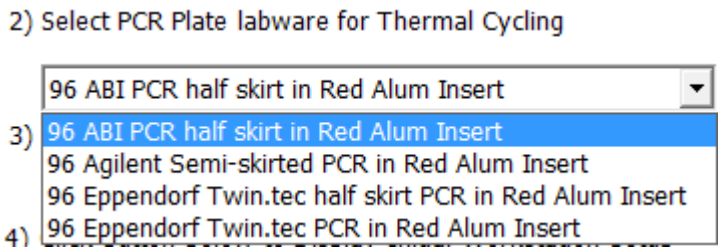


Table 12 Ordering information for supported PCR plates

Description in VWorks menu	Vendor and part number
96 ABI PCR half-skirted plates (MicroAmp Optical plates)	Life Technologies 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 or 951020619

3 Sample Preparation

This section contains instructions for indexed gDNA library preparation specific to the Illumina multiplexed, paired end sequencing platform and to automated processing using the Agilent NGS Workstation. For each sample to be sequenced, an individual indexed library is prepared. See the Reference chapter, starting on page 127 for sequences of the index portion of the indexing adaptors ligated to gDNA libraries in this section.

The steps in this section differ from the Illumina protocol in the use of the Covaris system for gDNA shearing, smaller target shear size, elimination of size selection by gel purification, implementation of AMPure XP beads (SPRI beads) for all purification steps, and primers used for PCR.

Refer to the Illumina protocol *Preparing Samples for Multiplexed Paired-End Sequencing* (p/n1005361) or the appropriate Illumina protocol for more information.

Step 1. Shear DNA

Before you begin, you can use the SureSelect gDNA Extraction Kit to extract genomic DNA. Refer to the *gDNA Extraction Kit Protocol* (p/n 5012-8701).

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

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.

   Follow the instructions for the instrument.

2. Dilute 1 µg 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 least 2 hours before use, 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 You can use the 96 microTube plate (see Table 4 on page 14) for the DNA shearing step when preparing multiple gDNA samples in the same experiment.

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 13 or Table 14, depending on the Covaris instrument SonoLab software version used.

The target DNA fragment size is 150 to 200 bp.

Table 13 Shear settings for Covaris instruments using SonoLab software version 7 or newer

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

Table 14 Shear settings for Covaris instruments using SonoLab software prior to version 7

Setting	Value
Duty Cycle	10%
Intensity	5
Cycles per Burst	200
Time	6 cycles of 60 seconds each
Set Mode	Frequency sweeping
Temperature	4° to 7° 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 50 µL of each sheared DNA sample to a separate well of a 96-well Eppendorf twin.tec 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. Assess sample quality and DNA fragment size

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*.

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 an average DNA fragment size of 150 to 200 bp. A sample electropherogram is shown in Figure 3.

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.

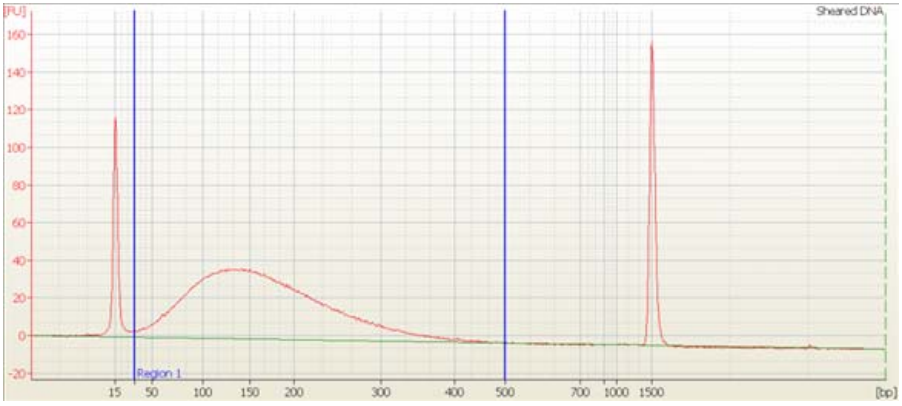


Figure 3 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay.

Option 2: Analysis using the Agilent 2200 TapeStation and D1000 ScreenTape

You can use Agilent's 2200 TapeStation for rapid analysis of multiple samples. Use a D1000 ScreenTape (p/n 5067-5582) and associated reagent kit (p/n 5067-5583) to analyze the sheared DNA. For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

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 *Agilent 2200 TapeStation 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 2200 TapeStation as instructed in the *Agilent 2200 TapeStation User Manual*. Start the run.

5 Verify that the electropherogram shows an average DNA fragment size of 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 sheared DNA sample plate and store at 4°C overnight or at –20°C for prolonged storage.

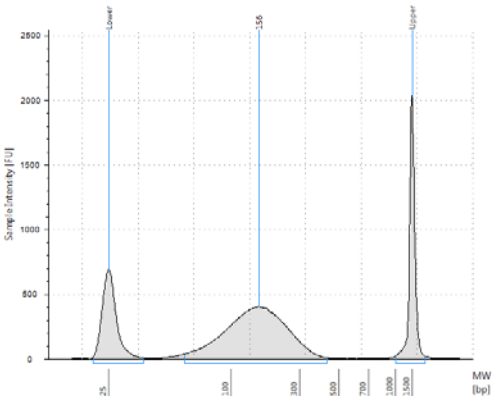


Figure 4 Analysis of sheared DNA using the 2200 TapeStation.

Step 3. Prepare indexed gDNA library samples

In this step, the Agilent NGS Workstation completes the DNA end modification steps required for SureSelect^{XT2} pre-capture indexing, including end-repair, A-tailing, and indexing adaptor ligation. Where required, the Agilent NGS Workstation purifies the prepared DNA using AMPure XP beads as part of the Library Prep runset.

Prepare the workstation

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

Prepare the Library Prep master mix source plate

4 In a Nunc DeepWell plate, prepare the master mix source plate by adding the volumes indicated in Table 15 of each reagent to all wells of the indicated column of the plate.

As indicated in the shaded portions of Table 15, Column 1 and Column 3 are prepared to contain mixtures of two reagents. Keep the reagents and source plate on ice during the aliquoting steps.

Table 15 Preparation of the Master Mix Source Plate for LibraryPrep_XT_Illumina_v2.0.rst

Reagent Solution	Position on Source Plate	Volume 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
SureSelect End Repair Enzyme Mix	Column 1 (A1-H1)	60 µL*	100 µL†	140 µL‡	180 µL**	260 µL††	520 µL‡‡
SureSelect End Repair Nucleotide Mix***		15 µL*	25 µL†	35 µL‡	45 µL**	65 µL††	130 µL‡‡
SureSelect dA-Tailing Master Mix	Column 2 (A2-H2)	30 µL	50 µL	70 µL	90 µL	130 µL	260 µL
SureSelect Ligation Master Mix	Column 3 (A3-H3)	7.5 µL	15.0 µL	20.0 µL	27.5 µL	40 µL	75 µL
Nuclease-free water		3.8 µL	7.5 µL	10.0 µL	13.8 µL	20 µL	37.5 µL

* For kits that include SureSelect End Repair Master Mix, add 75 µL of the pre-combined master mix for 1-column runs.

† For kits that include SureSelect End Repair Master Mix, add 125 µL of the pre-combined master mix for 2-column runs.

‡ For kits that include SureSelect End Repair Master Mix, add 175 µL of the pre-combined master mix for 3-column runs.

** For kits that include SureSelect End Repair Master Mix, add 225 µL of the pre-combined master mix for 4-column runs.

†† For kits that include SureSelect End Repair Master Mix, add 325 µL of the pre-combined master mix for 6-column runs.

‡‡ For kits that include SureSelect End Repair Master Mix, add 650 µL of the pre-combined master mix for 12-column runs.

***May also be labeled as *SureSelect End Repair Oligo Mix*.

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

6 Vortex the plate for 5 seconds to ensure homogeneity of the mixtures.

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

The final configuration of the master mix source plate is shown in Figure 5.

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.

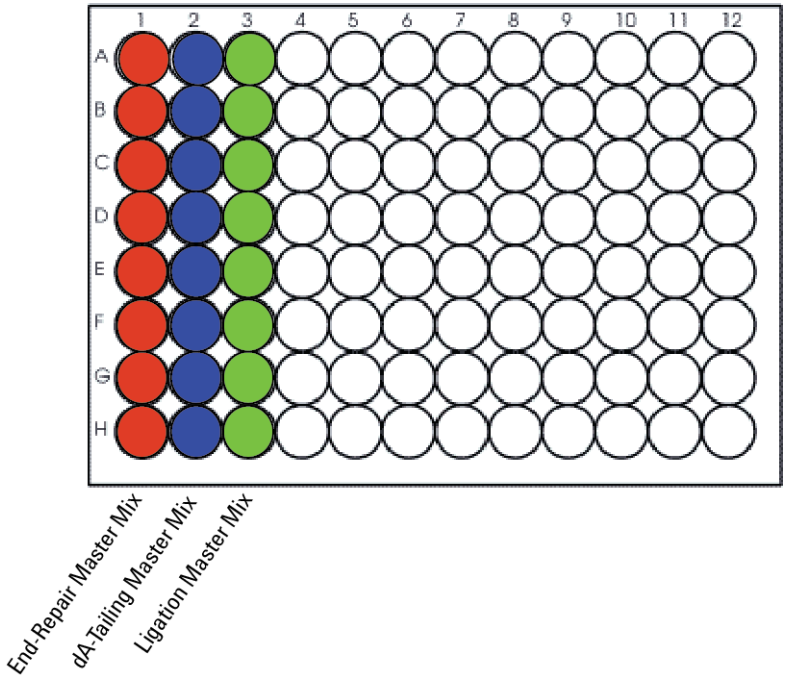


Figure 5 Configuration of the master mix source plate for LibraryPrep_XT_Illumina_v2.0.rst

Prepare the Pre-capture Indexed Adaptors source plate

8 Select the appropriate index for each sample. Nucleotide sequence information for the index portion of each indexed adaptor is provided in the Reference chapter, starting on page 127.

Using an Eppendorf Twin.tec plate, prepare the indexed adaptors source plate by combining 5 µL of each SureSelect Pre-capture Indexed Adaptor solution with 2.5 µL of nuclease-free water. Each pre-capture index dilution is made in a separate well of the source plate, corresponding to the well position of the sample to be indexed.

Prepare the purification reagents

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

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

11 Prepare a separate Nunc DeepWell source plate for the beads by adding 250 µL of homogeneous AMPure XP beads per well, for each well to be processed.

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

13 Prepare a separate Thermo Scientific reservoir containing 100 mL of freshly-prepared 70% ethanol.

Load the Agilent NGS Workstation

14 Load the Labware MiniHub according to Table 16, using the plate orientations shown in Figure 6.

Table 16 Initial MiniHub configuration for LibraryPrep_XT_Illumina_v2.0.rst

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty	Empty Nunc DeepWell plate	Empty Nunc DeepWell plate	Indexing Adaptors in Eppendorf twin.tec plate
Shelf 4	Empty	Empty Eppendorf twin.tec plate	Empty Eppendorf twin.tec plate	Empty
Shelf 3	Empty	Empty	Empty Eppendorf twin.tec plate	Empty
Shelf 2	Empty tip box	Nuclease-free water reservoir from step 12	AMPure XP beads in Nunc DeepWell plate from step 11	Empty
Shelf 1 (Bottom)	New tip box	70% ethanol reservoir from step 13	Empty	Empty tip box

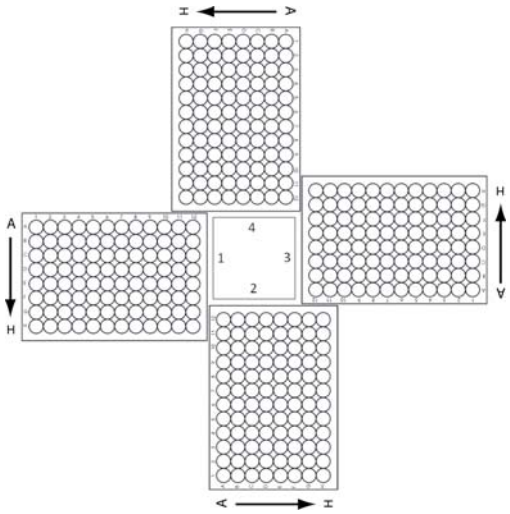


Figure 6 Labware MiniHub plate orientation. For Thermo Scientific reservoirs, place the notched corner facing the center of the hub.

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

Table 17 Initial BenchCel configuration for LibraryPrep_XT_Illumina_v2.0.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	11 Tip boxes	3 Tip boxes	Empty	Empty

16 Load the Bravo deck according to Table 18.

Table 18 Initial Bravo deck configuration for LibraryPrep_XT_Illumina_v2.0.rst

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
6	Empty Eppendorf twin.tec plate
7	Eppendorf twin.tec plate containing sheared gDNA samples, oriented with well A1 in the upper-left
9	Library Prep Master Mix Source Plate (unsealed) seated on silver insert

Run VWorks runset LibraryPrep_XT_Illumina_v2.0.rst

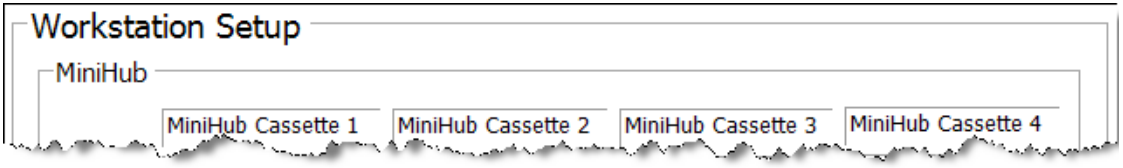
NOTE For this runset, you are not required to select PCR Plate labware under step 2 on the setup form.

17 On the SureSelect setup form, under **Select Protocol to Run**, select **LibraryPrep_XT_Illumina_v2.0.rst.**

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

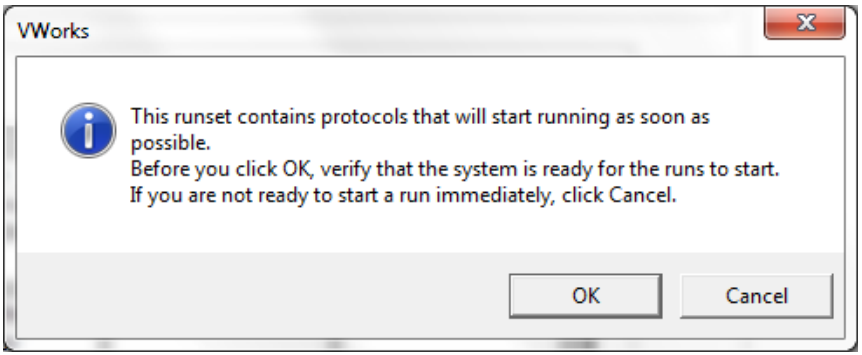
19 Click **Display Initial Workstation Setup**.

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



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

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



Running the LibraryPrep_XT_Illumina_v2.0.rst runset takes approximately 3 hours. Once complete, the purified, indexing adaptor-ligated DNA samples are located in the Eppendorf twin.tec 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 the indexed libraries

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

In this protocol, one half of the 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. Leave tip boxes on shelves 1 and 2 in cassette 1 of the Labware MiniHub from the previous LibraryPrep_XT_Illumina_v2.0.rst run. Otherwise, clear the remaining positions of the MiniHub and BenchCel of plates and tip boxes.
2. 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.
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 the master mix source plate

4 Prepare the Pre-capture PCR Master Mix by combining SureSelect Herculase II Master Mix and the XT2 Primer Mix in column 4 of the master mix source plate. Add the volumes of both reagents shown in Table 19 to each well of column 4 of the master mix source plate.

Use the same Nunc DeepWell master mix source plate that was used for the LibraryPrep_XT_Illumina_v2.0.rst run. The final configuration of the master mix source plate is shown in Figure 7.

Table 19 Preparation of the Master Mix Source Plate for Pre-CapturePCR_XT_Illumina_v2.0.pro

SureSelect^{XT2} Reagent	Position on Source Plate	Volume of Reagents 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
SureSelect Herculase II Master Mix	Column 4 (A4-H4)	37.5 µL	62.5 µL	87.5 µL	112.5 µL	162.5 µL	325 µL
XT2 Primer Mix		1.5 µL	2.5 µL	3.5 µL	4.5 µL	6.5 µL	13.0 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 indexing adaptor-ligated DNA sample), leave columns 1 to 3 empty and add the PCR Master Mix to column 4 of the new plate.

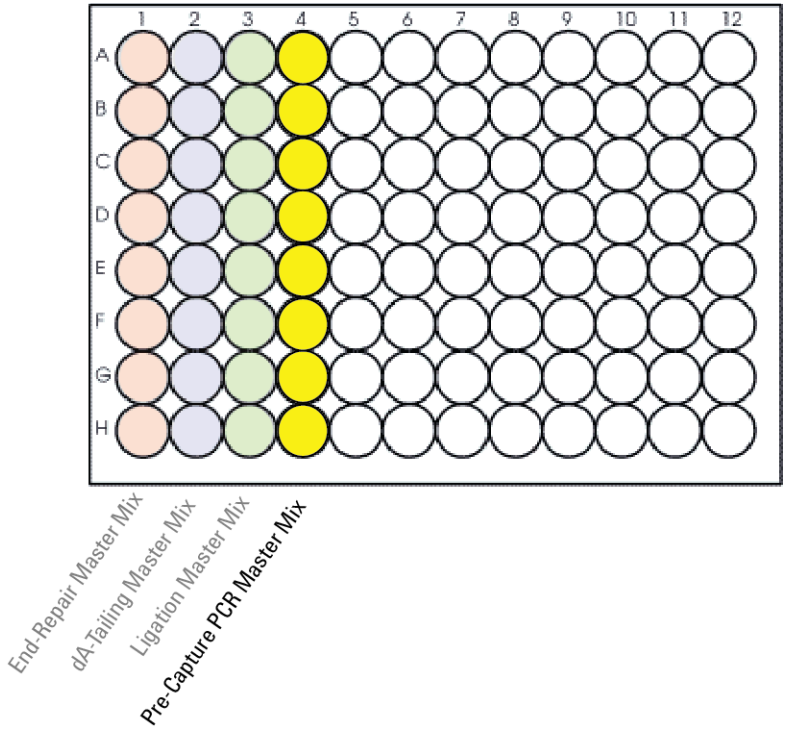


Figure 7 Configuration of the master mix source plate for Pre-CapturePCR_XT_Illumina_v2.0.pro. Columns 1-3 were used to dispense master mixes during the previous protocol.

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

6 Vortex the plate for 5 seconds to ensure homogeneity of the PCR Master Mix.

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

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

Load the Agilent NGS Workstation

8 Load the Labware MiniHub according to Table 20, using the plate orientations shown in Figure 6.

The waste tip box (Cassette 1, Shelf 2) and clean tip box (Cassette 1, Shelf 1) are retained from the LibraryPrep_XT_Illumina_v2.0.rst run and reused here. If you are using a new box of tips on shelf 1 of cassette 1 (for example, when amplifying the second half of the indexed DNA sample), first remove the tips from columns 1 to 3 of the tip box.

CAUTION Any tips present in columns 1 to 3 of the clean tip box (Cassette 1, Shelf 1) may be inappropriately loaded onto the Bravo platform pipette heads and may interfere with automated processing steps.

Table 20 Initial MiniHub configuration for Pre-CapturePCR_XT_Illumina_v2.0.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

* Retained from the LibraryPrep_XT_Illumina_v2.0.rst run and reused here.

9 Load the BenchCel Microplate Handling Workstation according to Table 21.

Table 21 Initial BenchCel configuration for Pre-CapturePCR_XT_Illumina_v2.0.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

10 Load the Bravo deck according to Table 22.

Table 22 Initial Bravo deck configuration for Pre-CapturePCR_XT_Illumina_v2.0.pro

Location	Content
6	Empty PCR plate seated on red insert (PCR plate type must be specified on setup form under step 2)
7	Indexing adaptor-ligated DNA samples, in Eppendorf twin.tec plate
9	Master mix plate (unsealed) containing Pre-Capture PCR Master Mix in Column 4 seated on silver insert

Run VWorks protocol Pre-CapturePCR_XT_Illumina_v2.0.pro

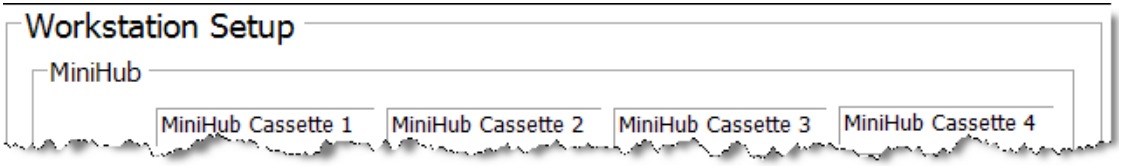
11 On the SureSelect setup form, under **Select Protocol to Run**, select **Pre-CapturePCR_XT_Illumina_v2.0.pro.**

12 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate that was loaded on Bravo deck position 6.

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_Illumina_v2.0.pro protocol takes approximately 15 minutes. Once complete, the PCR-ready samples, containing indexed 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 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 23.

The volume of each PCR amplification reaction is 50 µL.

Table 23 Pre-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	5	98°C	30 seconds
		60°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 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.)
3. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
4. Prepare a Nunc DeepWell source plate for the beads by adding 65 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 24, using the plate orientations shown in Figure 6.

Table 24 MiniHub configuration for SPRI_XT_Illumina_v2.0.pro:Pre-Capture PCR Cleanup

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 twin.tec 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 BenchCel Microplate Handling Workstation according to Table 25.

Table 25 BenchCel configuration for SPRI_XT_Illumina_v2.0.pro:Pre-Capture PCR Cleanup

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

9 Load the Bravo deck according to Table 26.

Table 26 Bravo deck configuration for SPRI_XT_Illumina_v2.0.pro:Pre-Capture PCR Cleanup

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)

Run VWorks protocol SPRI_XT_Illumina_v2.0.pro:Pre-Capture PCR Cleanup

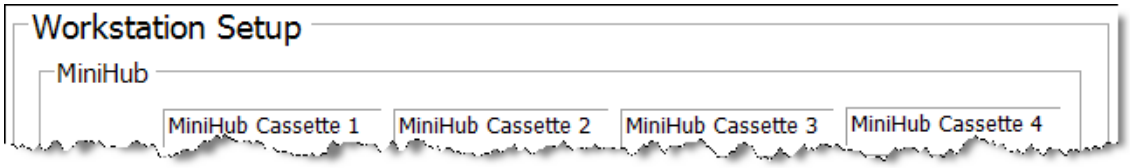
10 On the SureSelect setup form, under **Select Protocol to Run**, select **SPRI_XT_Illumina_v2.0.pro:Pre-Capture PCR Cleanup.**

11 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate that was loaded on Bravo deck 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 amplified DNA samples are in the Eppendorf plate located on Bravo deck position 7.

Step 6. Assess Library DNA quantity and quality

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*.

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 an average DNA amplicon size of 250 to 300 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.

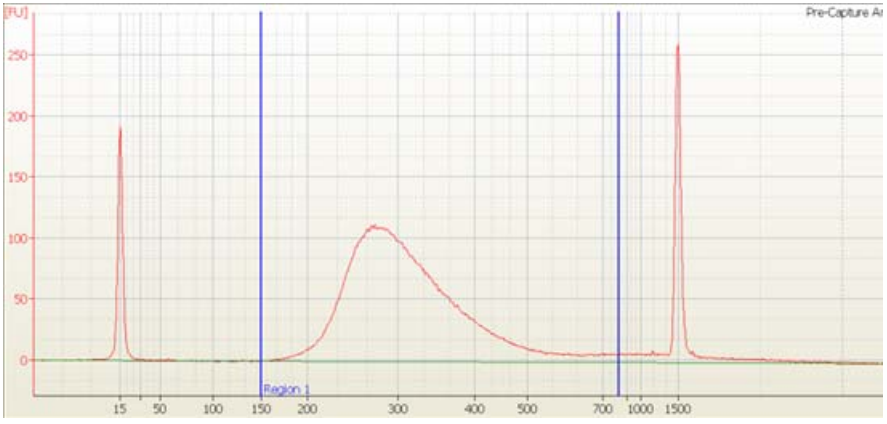


Figure 8 Analysis of amplified prepped library DNA using a DNA 1000 assay.

Option 2: Analysis using the Agilent 2200 TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape (p/n 5067-5582) and associated reagent kit (p/n 5067-5583) to analyze the amplified prepped DNA. For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

1. Seal the DNA library 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 *Agilent 2200 TapeStation User Manual*. Use 1 µL of each 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 2200 TapeStation as instructed in the *Agilent 2200 TapeStation User Manual*. Start the run.
5. Verify that the electropherogram shows an average DNA amplicon size of 250 to 300 bp. A sample electropherogram is shown in Figure 9.
6. Determine the DNA concentration (ng/µL) by integrating under the peak.

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.

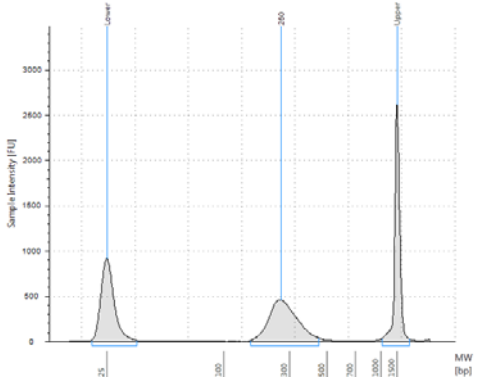


Figure 9 Analysis of amplified DNA using the 2200 TapeStation.

4 Hybridization

This chapter describes the steps to pool indexed gDNA libraries and then hybridize the pooled gDNA libraries with a SureSelect or ClearSeq Capture Library. Pools of 8 or 16 indexed samples are hybridized to the appropriate Capture Library and the targeted molecules are captured for sequencing. See Table 27 for the recommended number of indexes per gDNA library pool for different types of Capture Libraries.

The size of your SureSelect or ClearSeq Capture Library determines the post-capture amplification cycle number. See Table 57 for cycle number recommendations for different Capture Library sizes. Plan your experiments for capture using similar-sized Capture Libraries on the same plate to facilitate post-capture amplification.

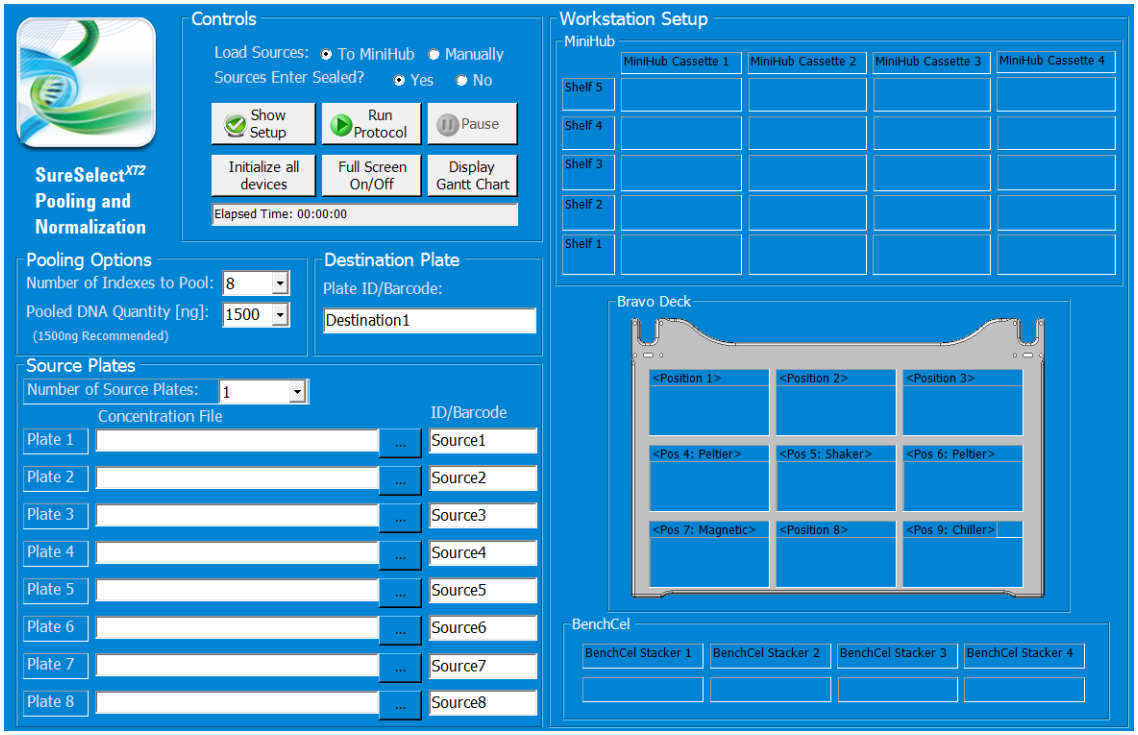
CAUTION The ratio of Capture Library to indexed gDNA library is critical for successful capture.

CAUTION You must avoid evaporation from the small volumes of the capture during the 24 hour or greater incubation.

If you want to use a duration of hybridization >24 hours, first test the conditions. Incubate 60 µL of SureSelect XT2 Hybridization Buffer (without DNA) at 65°C for 24 hours (or longer, if applicable) as a test. Include buffer in each well that you might use, including those in the center and those on the edges. Check that you do not get extensive evaporation. Evaporation should not exceed 6 to 8 µL.

Step 1. Pool indexed DNA samples for hybridization

In this step, the workstation pools the prepped indexed gDNA samples, before hybridization to the SureSelect or ClearSeq Capture Library. This workflow step is set up using the VWorks Form XT2_Pooling.VWForm shown below.



Plan pooling run parameters

The Hybridization reaction requires 1500 ng indexed gDNA, made up of a pool containing equal amounts of 8 or 16 individual libraries. See Table 27 for the recommended pool composition based on your SureSelect or ClearSeq Capture Library.

Where possible, indexed DNA pools are prepared containing a total DNA amount of 1500 ng. For some indexed DNA pools, the initial library pool will contain >1500 ng DNA, as detailed below, with 1500 ng of the pooled DNA added to the Hybridization reaction at a later step.

Table 27 Pre-capture pooling of indexed DNA libraries

Capture Library	Number of indexed gDNA libraries per pool	Amount of each indexed gDNA library in pool
SureSelect Custom Capture Libraries	16	93.75 ng
ClearSeq Comprehensive Cancer	16	93.75 ng
ClearSeq DNA Kinome	16	93.75 ng
SureSelect Human or Mouse All-Exon	8	187.5 ng
SureSelect Clinical Research Exome	8	187.5 ng
SureSelect Focused Exome	8	187.5 ng
ClearSeq Inherited Disease	8	187.5 ng

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

Accurate normalization of pools requires a minimum pipetting volume of 2 µL for each sample. Maximum DNA concentration values for a 1500 ng pool containing >2 µL of each sample are shown in Table 27, above. When higher-concentration DNA samples are included in the pooling run, the DNA pool amount must be adjusted as described below.

1 Check the DNA concentration of each sample in the set of source plates to be pooled to a single daughter plate to determine the appropriate amount of DNA per pool.

   a If all samples contain DNA at concentrations below the maximum DNA concentration shown in Table 27 (<94 ng/µL for All Exon and

Inherited Disease captures or at <47 ng/µL for DNA Kinome and custom captures), then prepare 1500 ng DNA pools.

b If at least one of the samples is above the maximum DNA concentration shown in Table 27 (>94 ng/µL for All Exon and Inherited Disease captures or at >47 ng/µL for DNA Kinome and custom captures), then you need to calculate the appropriate DNA pool amount. First, identify the most concentrated DNA sample and calculate the amount of DNA contained in 2 µL of that sample. This becomes the amount of each DNA sample used for pooling in the run. For example, if the highest DNA sample concentration is 100 ng/µL, then the final DNA pool will contain 200 ng of each indexed DNA. Next, determine the total amount of DNA per pool, based on the Capture Library size. Continuing with the same example, an All Exon capture pool would contain 8 × 200 ng, or 1600 ng DNA.

2 Determine the appropriate daughter plate type, based on DNA pool volumes. First, calculate the volume of each indexed DNA sample to be pooled, using the concentration values for each sample and the amount of each DNA sample per pool from step 1 above. Next, calculate the expected total pool volume for each indexed DNA pool included on the daughter plate.

a If the volume for all pools in the run is <180 µL, then use an Eppendorf twin.tec plate as the daughter (destination) plate for the pooling protocol. This plate will be used directly as indexed DNA pool source plate in the Hybridization protocol.

b If the volume for any pool in the run is >180 µL, then use a Nunc DeepWell plate as the daughter (destination) plate for the pooling protocol. After pool volumes are standardized (see page 71) the indexed DNA pools must be transferred to an Eppendorf twin.tec plate for the Hybridization protocol.

Plan daughter indexed DNA pool sample plate configuration

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

- When using a single Capture Library for all wells on the plate, fill the plate column-wise in well order A1 to H1, then A2 to H2, ending with A12 to H12.

- When using multiple Capture Libraries, configure the plate such that all gDNA library pools to be hybridized to a particular Capture Library are positioned in appropriate rows or columns. When using the Hybridization_MMCol_v2.0.pro protocol, place samples to be enriched using the same library in the same **row**. When using the Hybridization_MMRow_v2.0.pro protocol, place samples to be enriched using the same library in the same **column**.
- Each 96-reaction library preparation run produces 6 or 12 gDNA pools. For greatest efficiency of reagent use, gDNA pools from multiple library preparation runs may be placed on the same daughter plate for hybridization.

Prepare .csv files for pooling and normalization

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

See Figure 10 for required .csv file content. Pooling and normalization .csv file templates are provided in the following directory:

C: > VWorks Workspace > NGS Option B > XT_Illumina_2.0 > Pooling and Normalization Templates

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

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

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

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

Figure 10 Sample pooling and normalization .csv file content

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

- In the **PreCap Amplified pond concentrations** field, enter the concentration (in ng/µL) determined on page 61 for each indexed DNA sample.
- In the **Target WellID field**, enter the well position of the pool in which the indexed DNA sample should be included for the Hybridization plate. See the guidelines on page 65 for Hybridization sample pool placement considerations.

Set up and run the PreCapture_Pooling_v1.0.pro automation protocol

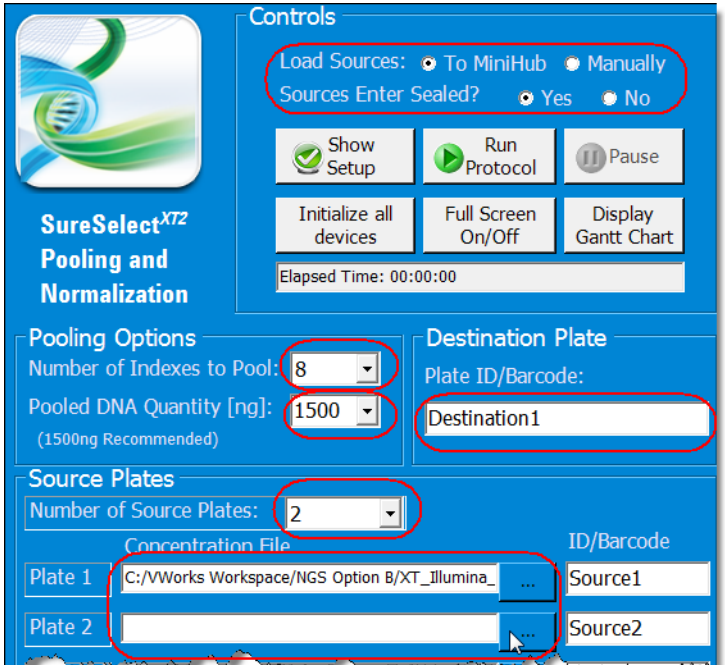
1 Turn on the chiller, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.

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

3 To set up the PreCapture_Pooling_v1.0.pro automation protocol, open the VWorks Form XT2_Pooling.VWForm using the shortcut on your desktop.

4 In the Form, enter the run information highlighted below:

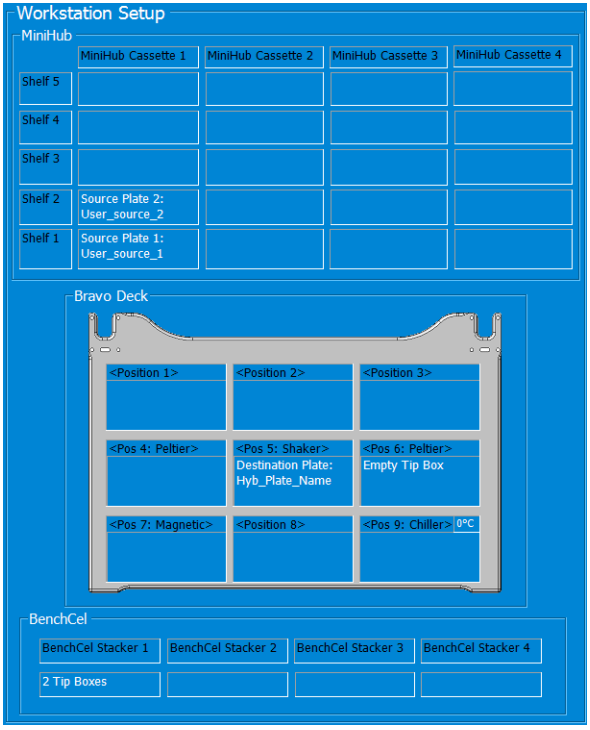
- Under **Controls**, specify whether the indexed DNA source plates will be loaded in the MiniHub and will be sealed at start of run (recommended).
- From **Number of Indexes to Pool** menu, select 8 or 16 (see Table 27 for guidelines).
- From **Pooled DNA Quantity** menu, enter the required total amount of DNA in the pool (typically 1500 ng). See page 65 for guidelines.
- In **Plate ID/Barcode** field, enter the name or barcode of the daughter Hybridization sample plate.
- From **Number of Source Plates** menu, select the number of indexed DNA source plates to be provided for sample pooling. If >8 plates will be used to create a single Hybridization sample plate, run the pooling and normalization protocol in sets of 8 source plates.
- Under **Concentration File**, use the browse button to specify the location of each .csv file that provides sample position and concentration data for each plate.



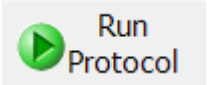
5 When finished entering run parameters in the Form, click **Show Setup**.

6 Load sample plates and labware as displayed in the Workstation Setup region of the form (example shown below is for pooling run for two source plates):

- Load each indexed DNA source plates onto its assigned shelf on the MiniHub.
- Load the appropriate type of destination (daughter) plate on Bravo deck position 5. See step 2 on page 66 to determine plate type needed.
- Load an empty tip box on Bravo deck position 6.
- Load the indicated number of tip boxes in the BenchCel stacker.



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



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

Running the PreCapture_Pooling_v1.0.pro protocol takes approximately one hour per indexed DNA source plate. Once complete, the Hybridization sample plate, containing indexed DNA pools, is located at position 5 of the Bravo deck.

Adjust final concentration of pooled DNA

8 Remove the Hybridization sample plate from Bravo deck position 5.

9 Use a vacuum concentrator, held at ≤ 45°C, to reduce the volume in each well to 1–2 µL.

10 Add sufficient nuclease-free water to each concentrated gDNA pool to bring the final DNA concentration to 214.3 ng/µL. For example, for 1500 ng pools, bring the final volume in each well to 7 µL, for a final concentration of 214.3 ng/µL.

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

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

13 If indexed DNA pool samples are in a Nunc DeepWell plate, carefully transfer the samples to an Eppendorf twin.tec plate for use in the following Hybridization protocol.

Step 2. Hybridize the gDNA library and Capture Library

In this step, the Agilent NGS Workstation completes the liquid handling steps in preparation for hybridization of the indexed DNA pools to one or more SureSelect or ClearSeq Capture Libraries. Afterward, you transfer the sample plate to a thermal cycler, held at 65°C, to allow hybridization of the indexed DNA to the Capture Library.

Two versions of the Hybridization automation protocol are available. See Table 28 for a summary of suggested usage and to locate the instructions for each protocol option in this manual.

For runs in which all samples will be hybridized to the same Capture Library, hybridization protocol selection is based on run size, where 12-column runs should use the Hybridization_MMCol_v2.0.pro protocol and 1-6 column runs should use the Hybridization_MMRow_v2.0.pro protocol.

For runs that include hybridization to multiple Capture Libraries, hybridization protocol selection is based on the following considerations:

1 Number of Capture Libraries

2 Appropriate positioning (rows vs. columns) of the Capture Libraries with respect to the DNA sample plate configuration:

- In Hybridization_MMCol_v2.0.pro, master mixes are organized in the source plate by column (see Figure 11) and each **row** of the DNA sample plate may be hybridized to a different Capture library
- In Hybridization_MMRow_v2.0.pro, master mixes are organized in the source plate by row (see Figure 12), and each **column** of the DNA sample plate may be hybridized to a different Capture library

Table 28 Comparison of Hybridization protocol options

Protocol Name	Optimal Hybridization Run Size	Number of Different Capture Libraries Allowed in Run	Instructions Start
Hybridization_MMCol_v2.0.pro	12-column runs	8	page 73
Hybridization_MMRow_v2.0.pro	≤ 6-column runs	12	page 86

Hybridization Option A: Master Mixes in Columns (Hybridization_MMCol_v2.0.pro)

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 NucleoClean decontamination wipe.

Prepare one or more Capture Library Master Mixes

3 Prepare the appropriate volume of SureSelect or ClearSeq Capture Library Master Mix for each of the Capture Libraries that will be used for hybridization as indicated in Table 29 to Table 32. Mix the components by pipetting. Keep the master mixes on ice during preparation and aliquoting.

NOTE

Each row of the indexed gDNA pool 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 29 or Table 30) below.

For runs that use different Capture Libraries for individual rows, prepare each master mix as described in Step b (Table 31 or Table 32) below.

a **For runs that use a single Capture Library for all rows**, prepare the Capture Library Master Mix as listed in Table 29 or Table 30, based on the Mb target size of your design.

Table 29 Preparation of Capture Library Master Mix for target sizes <3.0 Mb; same Capture Library for all 8 rows of wells

Target size <3.0 Mb		
SureSelect^{XT2} Reagent	**Volume for 1 Well**	**Volume for 12 Columns of Wells**
Nuclease-free water	7.0 µL	808.5 µL
SureSelect RNase Block (purple cap)	0.5 µL	57.8 µL
SureSelect or ClearSeq Capture Library	2.0 µL	231.0 µL
Total Volume	**9.5 µL**	**1097.3 µL**

Table 30 Preparation of Capture Library Master Mix for target sizes >3.0 Mb; same Capture Library for all 8 rows of wells

Target size >3.0 Mb		
SureSelect^{XT2} Reagent	**Volume for 1 Well**	**Volume for 12 Columns of Wells**
Nuclease-free water	4.0 µL	462.0 µL
SureSelect RNase Block (purple cap)	0.5 µL	57.8 µL
SureSelect or ClearSeq Capture Library	5.0 µL	577.5 µL
Total Volume	**9.5 µL**	**1097.3 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 31 or Table 32, based on the Mb target size of your design. The volumes listed in Table 31 and Table 32 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 31 Preparation of Capture Library Master Mix for target sizes <3.0 Mb, single row of wells

Target size <3.0 Mb							
SureSelect^{XT2} Reagent	**Volume for 1 Well**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	7.0 µL	14.0 µL	21.2 µL	28.4 µL	35.7 µL	53.7 µL	100.6 µL
SureSelect RNase Block (purple cap)	0.5 µL	1.0 µL	1.5 µL	2.0 µL	2.5 µL	3.8 µL	7.2 µL
Capture Library	2.0 µL	4.0 µL	6.1 µL	8.1 µL	10.2 µL	15.3 µL	28.8 µL
Total Volume	**9.5 µL**	**19.0 µL**	**28.8 µL**	**38.6 µL**	**48.4 µL**	**72.9 µL**	**136.6 µL**

Table 32 Preparation of Capture Library Master Mix for target sizes >3.0 Mb, single row of wells

Target size >3.0 Mb							
SureSelect^{XT2} Reagent	**Volume for 1 Well**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.0 µL	8.0 µL	12.1 µL	16.3 µL	20.4 µL	30.7 µL	57.5 µL
SureSelect RNase Block (purple cap)	0.5 µL	1.0 µL	1.5 µL	2.0 µL	2.5 µL	3.8 µL	7.2 µL
Capture Library	5.0 µL	10.0 µL	15.2 µL	20.3 µL	25.5 µL	38.4 µL	71.9 µL
Total Volume	**9.5 µL**	**19.0 µL**	**28.8 µL**	**38.6 µL**	**48.4 µL**	**72.9 µL**	**136.6 µL**

Prepare the master mix source plate

4 In a Nunc DeepWell plate, prepare the hybridization master mix source plate at room temperature. Add the volumes indicated in Table 33 to all wells of the indicated column of the Nunc DeepWell plate. As indicated in the shaded portion of Table 33, Blocking Mix and nuclease-free water are combined in the wells of Column 1.

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 11.

Table 33 Preparation of the Master Mix Source Plate for Hybridization_MMCol_v2.0.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
SureSelect XT2 Blocking Mix	Column 1 (A1-H1)	13.5 µL	22.5 µL	31.5 µL	40.5 µL	58.5 µL	117.0 µL
Nuclease-free water		3.75 µL	6.25 µL	8.75 µL	11.25 µL	16.25 µL	32.5 µL
Capture Library Master Mix	Column 2 (A2-H2)	18.4 µL	28.2 µL	38.0 µL	47.8 µL	72.3 µL	136.0 µL
SureSelect XT2 Hybridization Buffer	Column 3 (A3-H3)	55.5 µL	92.5 µL	129.5 µL	166.5 µL	240.5 µL	481 µL

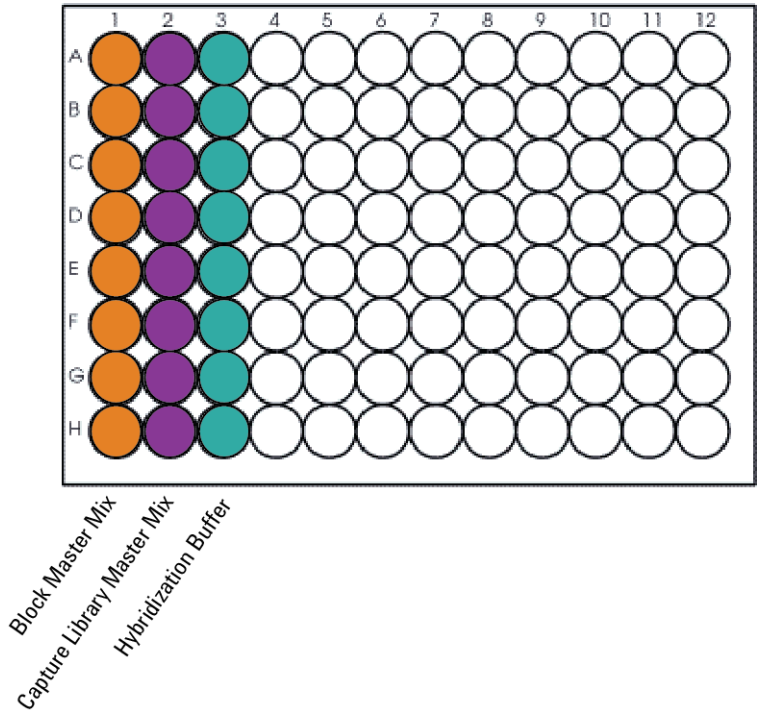


Figure 11 Configuration of the master mix source plate for Hybridization_MMCol_v2.0.pro. Each well in column 2 may contain the same or different Capture Libraries.

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

6 Vortex the plate for 5 seconds to ensure homogeneity of the Block Master Mix dilution.

7 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

8 Load the Labware MiniHub according to Table 34, using the plate orientations shown in Figure 6.

Table 34 Initial MiniHub configuration for Hybridization_MMCol_v2.0.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

9 Load the BenchCel Microplate Handling Workstation according to Table 35.

Table 35 Initial BenchCel configuration for Hybridization_MMCol_v2.0.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

10 Load the Bravo deck according to Table 36.

Table 36 Initial Bravo deck configuration for Hybridization_MMCol_v2.0.pro

Location	Content
4	Empty PCR plate seated on red insert (PCR plate type must be specified on setup form under step 2)
5	Empty Eppendorf twin.tec plate
6	Hybridization Master Mix source plate (unsealed) seated on silver insert (Master Mixes in Columns 1-3)
8	Empty tip box
9	Indexed DNA pools in Eppendorf twin.tec plate (unsealed)

Run VWorks protocol Hybridization_MMCol_v2.0.pro

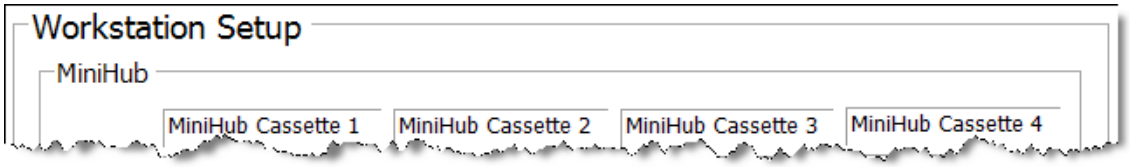
11 On the SureSelect setup form, under **Select Protocol to Run**, select **Hybridization_MMCol_v2.0.pro.**

12 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate that was loaded on Bravo deck position 4.

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**.

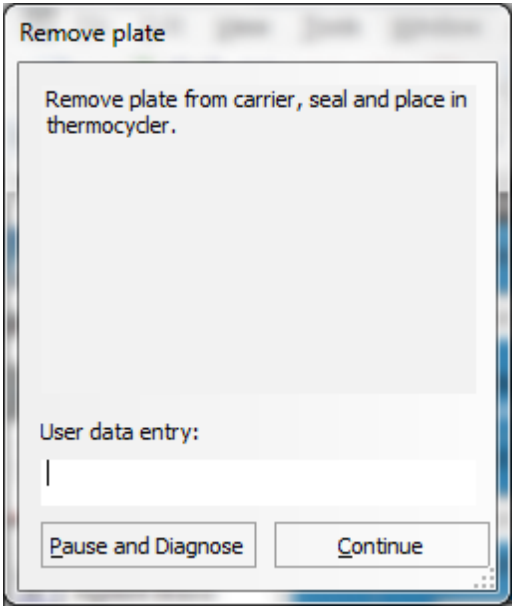
15 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

The Agilent NGS Workstation transfers Blocking Mix and indexed gDNA pools to the PCR plate. When this process is complete, you will be prompted to transfer the plate to the thermal cycler for sample denaturation prior to hybridization.

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



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

19 Transfer the sealed plate to a thermal cycler and run the following program shown in Table 37. After transferring the plate, click **Continue** on the VWorks screen.

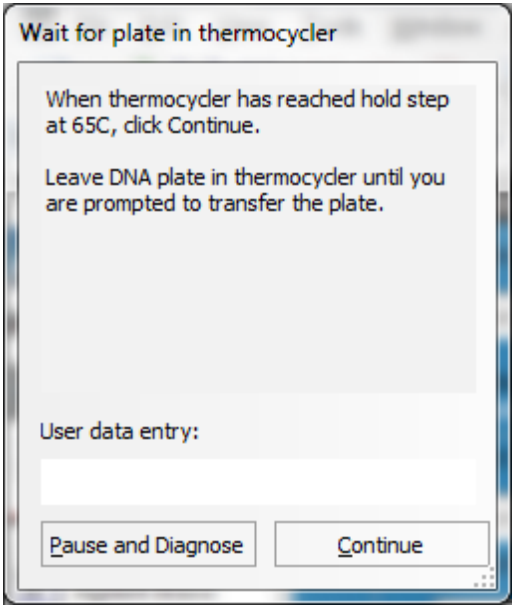
Table 37 Thermal cycler program used for sample denaturation prior to hybridization

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

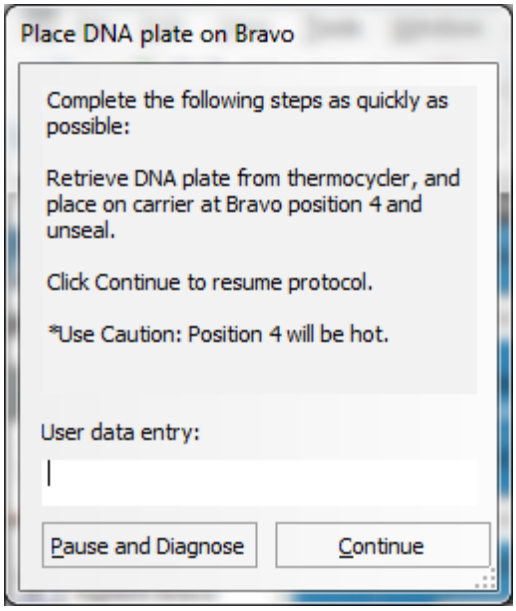
While the sample plate incubates on the thermal cycler, the Agilent NGS Workstation combines aliquots of the Capture Library master mix and Hybridization Buffer.

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

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



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



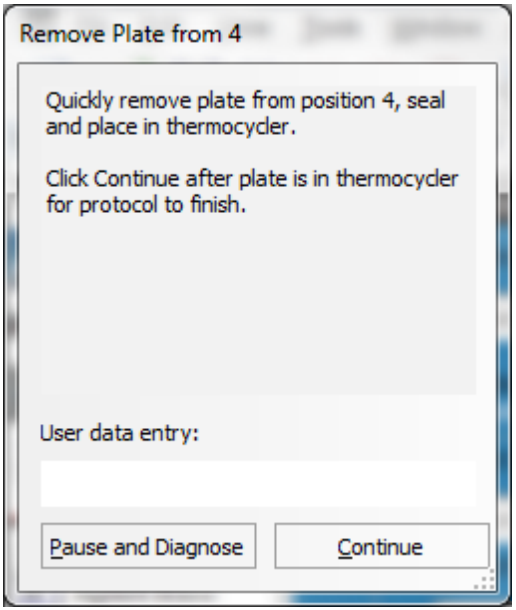
WARNING

Bravo deck position 4 will be hot.

Use caution when handling components that contact heated deck positions.

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

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



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

24 Quickly transfer the plate back to the thermal cycler, held at 65°C. Place a compression mat over the PCR plate in the thermal cycler. After transferring the plate, click **Continue** on the VWorks screen.

25 To finish the VWorks protocol, click **Continue** in the **Unused Tips** and **Empty Tip box** dialogs, and then 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.

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

Samples may be hybridized for up to 72 hours, but you must verify that the extended hybridization does not cause extensive evaporation in the sample wells.

When hybridization is complete, proceed to "Step 3. Capture the hybridized DNA" on page 97.

Hybridization Option B: Master Mixes in Rows (Hybridization_MMRow_v2.0.pro)

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 NucleoClean decontamination wipe.

Prepare one or more Capture Library Master Mixes

3 Prepare the appropriate volume of Capture Library Master Mix for each of the Capture Libraries that will be used for hybridization as indicated in Table 38 to Table 41. Mix the components by pipetting. Keep the master mixes on ice during preparation and aliquoting.

NOTE

Each column of the indexed gDNA pool 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 columns of the plate, prepare the master mix as described in Step a (Table 38 or Table 39) below.

For runs that use different Capture Libraries for individual columns, prepare each master mix as described in Step b (Table 40 or Table 41) below.

a **For runs that use a single Capture Library for all columns**, prepare the Capture Library Master Mix as listed in Table 38 or Table 39, based on the Mb target size of your design.

Table 38 Preparation of Capture Library Master Mix for target sizes <3.0 Mb; same Capture Library for all columns

Target size <3.0 Mb						
SureSelect^{XT2} Reagent	**Volume for 1 Well**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**
Nuclease-free water	7.0 µL	68.3 µL	143.3 µL	211.6 µL	279.8 µL	416.3 µL
SureSelect RNase Block (purple cap)	0.5 µL	4.9 µL	10.2 µL	15.1 µL	20.0 µL	29.7 µL
Capture Library	2.0 µL	19.5 µL	41.0 µL	60.5 µL	80.0 µL	119.0 µL
Total Volume	**9.5 µL**	**92.7 µL**	**194.5 µL**	**287.1 µL**	**379.8 µL**	**565.0 µL**

Table 39 Preparation of Capture Library Master Mix for target sizes >3.0 Mb; same Capture Library for all columns

Target size >3.0 Mb						
SureSelect^{XT2} Reagent	**Volume for 1 Well**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**
Nuclease-free water	4.0 µL	39.0 µL	81.9 µL	120.9 µL	159.9 µL	237.9 µL
SureSelect RNase Block (purple cap)	0.5 µL	4.9 µL	10.2 µL	15.1 µL	20.0 µL	29.7 µL
Capture Library	5.0 µL	48.8 µL	102.4 µL	151.1 µL	199.9 µL	297.4 µL
Total Volume	**9.5 µL**	**92.7 µL**	**194.5 µL**	**287.1 µL**	**379.8 µL**	**565.0 µL**

b **For runs that use different Capture Libraries in individual columns**, prepare a Capture Library Master Mix for each Capture Library as listed in Table 40 or Table 41, based on the Mb target size of your design. The volumes listed in Table 40 and Table 41 are for a single column of sample wells. If a given Capture Library will be hybridized in multiple columns, multiply each of the values below by the number of columns assigned to that Capture Library.

Table 40 Preparation of Capture Library Master Mix for target sizes <3.0 Mb, single column of wells

Target size <3.0 Mb		
SureSelect^{XT2} Reagent	**Volume for 1 Well**	**Volume for 1 Column**
Nuclease-free water	7.0 µL	68.3 µL
SureSelect RNase Block (purple cap)	0.5 µL	4.9 µL
SureSelect or ClearSeq Capture Library	2.0 µL	19.5 µL
Total Volume	**9.5 µL**	**92.7 µL**

Table 41 Preparation of Capture Library Master Mix for target sizes >3.0 Mb, single column of wells

Target size >3.0 Mb		
SureSelect^{XT2} Reagent	**Volume for 1 Well**	**Volume for 1 Column**
Nuclease-free water	4.0 µL	39.0 µL
SureSelect RNase Block (purple cap)	0.5 µL	4.9 µL
SureSelect or ClearSeq Capture Library	5.0 µL	48.8 µL
Total Volume	**9.5 µL**	**92.7 µL**

Prepare the master mix source plate

4 In a Nunc DeepWell plate, prepare the hybridization master mix source plate at room temperature. Add the volumes indicated in Table 42 to the appropriate number of wells of the indicated row of the Nunc DeepWell plate. Fill the number of wells that corresponds to the number of DNA-sample columns in the run (1, 2, 3, 4, 6, or 12).

As indicated in the shaded portion of Table 42, Blocking Mix and nuclease-free water are combined in the wells of Row A.

When using multiple Capture Libraries in a run, add each Capture Library Master Mix to the appropriate column(s) of the Nunc DeepWell plate.

The final configuration of the master mix source plate is shown in Figure 12.

Table 42 Preparation of the Master Mix Source Plate for Hybridization_MMRow_v2.0.pro

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well
SureSelect XT2 Blocking Mix	Row A (A1-AX)	81.0 µL
Nuclease-free water		22.5 µL
Capture Library Master Mix	Row B (B1-BX)	92.7 µL
SureSelect XT2 Hybridization Buffer	Row C (C1-CX)	314.5 µL

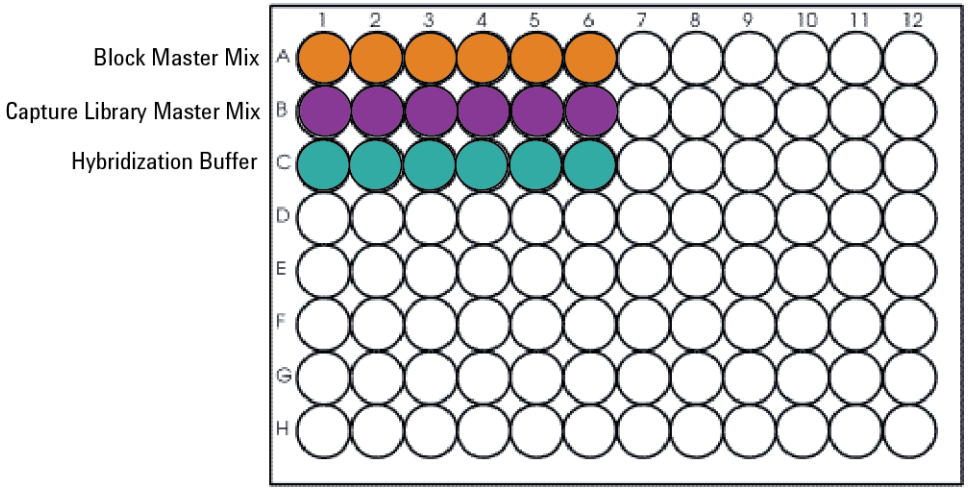


Figure 12 Configuration of the master mix source plate for Hybridization_MMRow_v2.0.pro. Rows A–C may contain 1, 2, 3, 4, 6, or 12 wells of reagents, depending on run size (example shown is for 6-column run size). Each well in row B may contain the same or different Capture Libraries.

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

6 Vortex the plate for 5 seconds to ensure homogeneity of the Block Master Mix dilution.

7 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

8 Load the BenchCel Microplate Handling Workstation according to Table 43.

Table 43 Initial BenchCel configuration for Hybridization_MMRow_v2.0.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	2 Tip boxes	Empty	Empty	Empty
4	2 Tip boxes	Empty	Empty	Empty
6	3 Tip boxes	Empty	Empty	Empty
12	5 Tip boxes	Empty	Empty	Empty

9 Load the Bravo deck according to Table 44.

Table 44 Initial Bravo deck configuration for Hybridization_MMRow_v2.0.pro

Location	Content
1	Empty tip box
4	Empty PCR plate seated on red insert (PCR plate type must be specified on setup form under step 2)
5	Empty Eppendorf twin.tec plate
6	Hybridization Master Mix source plate (unsealed) seated on silver insert (Master Mixes in Rows A-C)
9	Indexed DNA pools in Eppendorf twin.tec plate

Run VWorks protocol Hybridization_MMRow_v2.0.pro

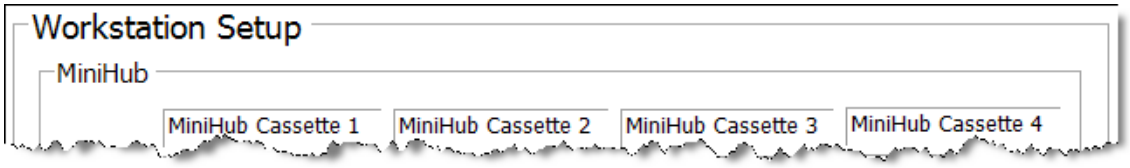
10 On the SureSelect setup form, under **Select Protocol to Run**, select **Hybridization_MMRow_v2.0.pro.**

11 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate that was loaded on Bravo deck position 4.

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

13 Click **Display Initial Workstation Setup**.

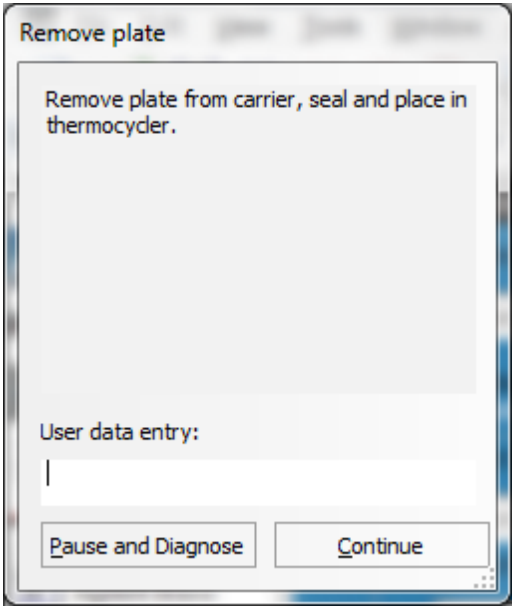
14 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

The Agilent NGS Workstation transfers Blocking Mix and indexed gDNA pools to the PCR plate. When this process is complete, you will be prompted to transfer the plate to the thermal cycler for sample denaturation prior to hybridization.

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



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

18 Transfer the sealed plate to a thermal cycler and run the following program shown in Table 37. After transferring the plate, click **Continue** on the VWorks screen.

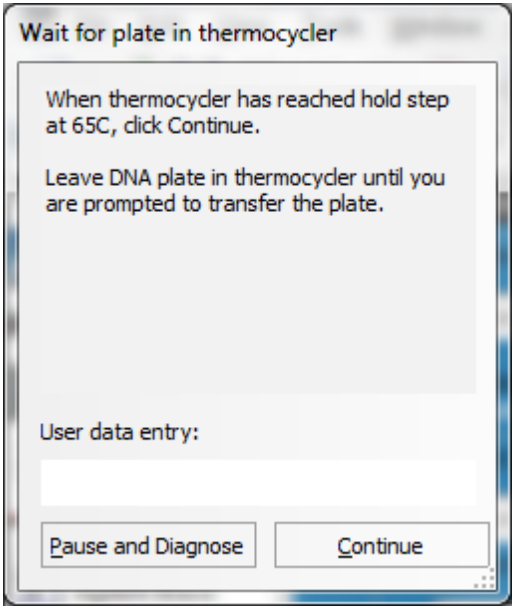
Table 45 Thermal cycler program used for sample denaturation prior to hybridization

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

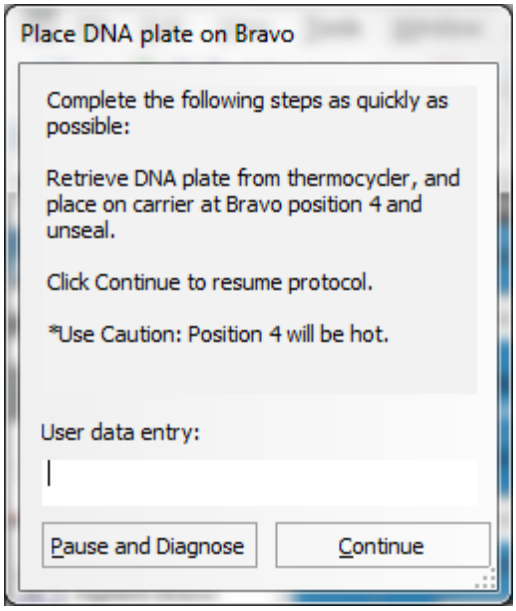
While the sample plate incubates on the thermal cycler, the Agilent NGS Workstation combines aliquots of the Capture Library master mix and Hybridization Buffer.

CAUTION You must complete step 19 to step 23 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.

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



20 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**.



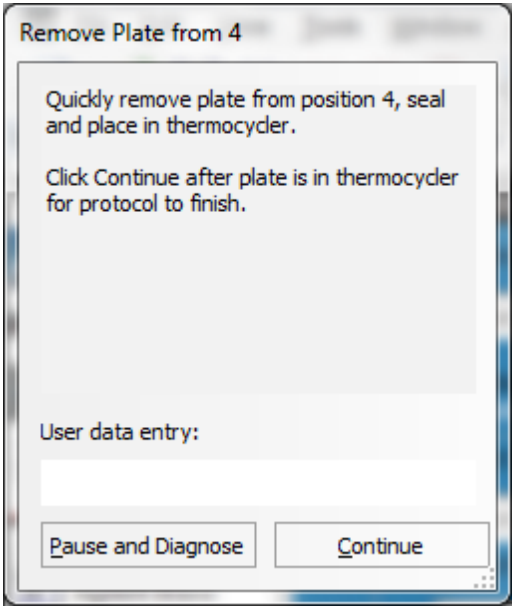
WARNING

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 indexed gDNA pools and blocking agents.

21 When prompted by VWorks as shown below, quickly remove the PCR sample plate from Bravo deck position 4, 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 Quickly transfer the plate back to the thermal cycler, held at 65°C. Place a compression mat over the PCR plate in the thermal cycler. After transferring the plate, click **Continue** on the VWorks screen.

24 To finish the VWorks protocol, click **Continue** in the **Unused Tips** and **Empty Tip box** dialogs, and then 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.

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

Samples may be hybridized for up to 72 hours, but you must verify that the extended hybridization does not cause extensive evaporation in the sample wells.

Step 3. Capture the hybridized DNA

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

This step is automated by the NGS workstation using the SureSelectCapture&Wash_v2.0.rst runset, with a total duration of approximately 2 hours. A workstation operator must be present to complete two actions during the runset, at the time points in the table below. The times provided are approximate; each action is completed in response to a VWorks prompt at the appropriate time in the runset.

Table 46

Operator action	Approximate time after run start
Transfer hybridization reaction plate from thermal cycler to NGS workstation	<5 minutes
Remove hybridization plate from position 4 after reactions transferred to capture plate	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 NucleoClean 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. On the Multi TEC control touchscreen, Bravo deck position 4 corresponds to CPAC 2, position 1.

Prepare the streptavidin-coated beads

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

5 Wash the magnetic beads.

a In a conical vial, combine the components listed in Table 47. The volumes below include the required overage.

Table 47 Components required for magnetic bead washing procedure

Reagent	Volume for 1 Well	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Streptavidin bead suspension	50 µL	425 µL	825 µL	1225 µL	1.65 mL	2.5 mL	5.0 mL
SureSelect XT2 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**	**4.7125mL**	**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 XT2 Binding Buffer.)

6 Resuspend the beads in SureSelect XT2 Binding Buffer, according to Table 48 below.

Table 48 Preparation of magnetic beads for SureSelect Capture&Wash_v2.0.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 XT2 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 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 an Eppendorf twin.tec source plate labeled *Wash #1*. For each well to be processed, add 160 µL of SureSelect XT2 Wash 1.

10 Prepare a Nunc DeepWell source plate labeled *Wash #2*. For each well to be processed, add 1150 µL of SureSelect XT2 Wash 2.

11 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 runset.

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

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

Load the Agilent NGS Workstation

14 Load the Labware MiniHub according to Table 49, using the plate orientations shown in Figure 6.

Table 49 Initial MiniHub configuration for SureSelect Capture&Wash_v2.0.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 twin.tec plate	Empty	*Wash #1* Eppendorf twin.tec source plate	Empty
Shelf 2	Empty	Nuclease-free water reservoir from step 13	Empty	Empty
Shelf 1 (Bottom)	Empty	Empty	Empty	Empty tip box

15 Load the BenchCel Microplate Handling Workstation according to Table 50.

Table 50 Initial BenchCel configuration for SureSelectCapture&Wash_v2.0.rst

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

16 Load the Bravo deck according to Table 51 (positions 5 and 6 should already be loaded).

Table 51 Initial Bravo deck configuration for SureSelectCapture&Wash_v2.0.rst

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
4	Empty red aluminum insert (PCR plate type used for Hybridization protocol must be specified on setup form under step 2)
5	Streptavidin beads DeepWell source plate
6	*Wash #2* DeepWell source plate seated on silver insert

Run VWorks runset SureSelectCapture&Wash_v2.0.rst

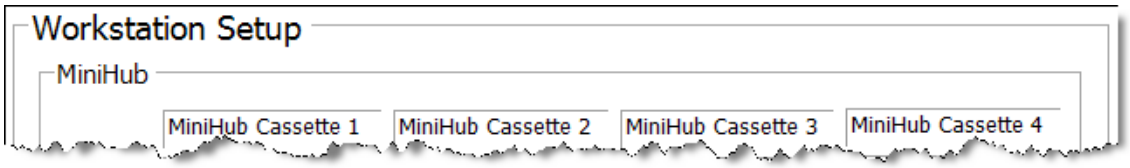
17 On the SureSelect setup form, under **Select Protocol to Run**, select **SureSelectCapture&Wash_v2.0.rst.**

18 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate used to incubate the hybridization reactions in the thermal cycler. This plate will be transferred from the thermal cycler to the NGS workstation in step 24 below.

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

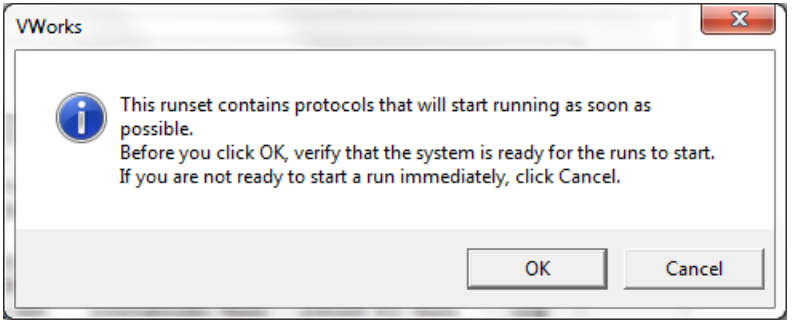
20 Click **Display Initial Workstation Setup**.

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



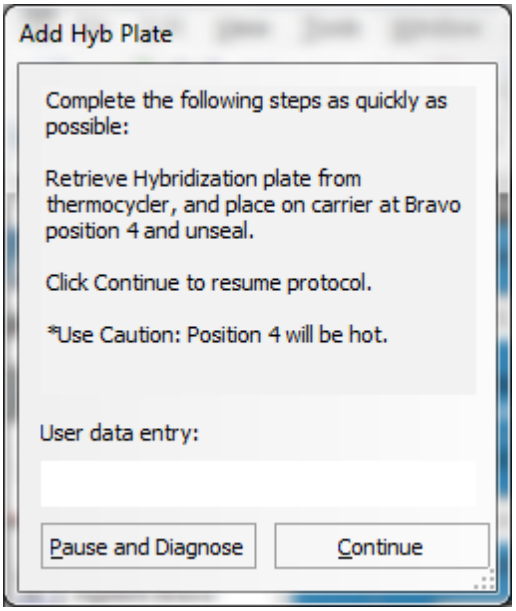
22 When verification is complete, click **Run Selected Protocol**.

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.

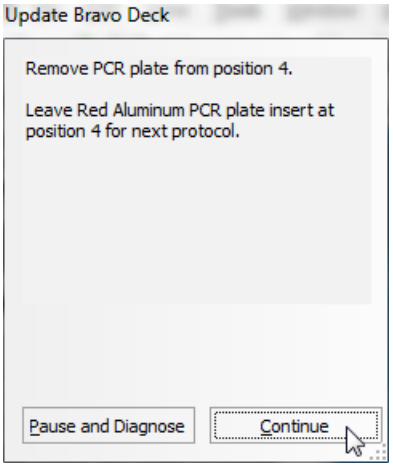
24 When prompted by VWorks as shown below, quickly remove the PCR plate, containing the hybridization reactions held at 65°C, from the thermal cycler. Unseal the plate carefully to avoid splashing, and quickly transfer the plate to position 4 of the Bravo deck, seated in the red aluminum insert. Click **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 the hybridization samples have been transferred from the PCR plate to the capture plate wells, you will be prompted by VWorks as shown below. Remove the PCR plate from position 4 of the Bravo deck, leaving the red insert in place. When finished, click **Continue** to resume the runset.



The remainder of the SureSelectCapture&Wash_v2.0.rst runset takes approximately 1.5 hours. Once the runset is complete, the captured, bead-bound DNA samples are located in the Eppendorf plate at position 9 of the Bravo deck.

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

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

5 Post-Capture Sample Processing for Multiplexed Sequencing

This chapter describes the steps to amplify, purify, and assess quality and quantity of the captured libraries. Post-capture dilution and optional pooling instructions are provided to prepare the indexed samples for multiplexed sequencing.

Step 1. Amplify the captured libraries

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

Plan your experiments for amplification of libraries captured using Capture Libraries of similar sizes on the same plate. See Table 57 for cycle number recommendations for different Capture Library size ranges.

Prepare the workstation

1. Clear the Labware MiniHub and BenchCel of plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo decks, and BenchCel with a Nucleoclean 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. 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 Post-capture PCR master mix source plate

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

The post-capture PCR master mix source plate must be a Nunc DeepWell plate, with the PCR master mix for the run supplied in column 4.

If the Hybridization protocol was run with master mixes configured by column (Hybridization_MMCol_v2.0.pro), reuse the Nunc DeepWell master mix source plate used for the Hybridization run. The final configuration of the master mix source plate for this scenario is shown in Figure 13.

If the Hybridization protocol was run with master mixes configured by row (Hybridization_MMRow_v2.0.pro), use a new Nunc DeepWell plate.

5 Prepare the Post-capture PCR Master Mix by combining SureSelect Herculase II Master Mix and the XT2 Primer Mix in column 4 of the master mix source plate. Add the volumes of both reagents shown in Table 52 to each well of column 4 of the master mix source plate.

Table 52 Preparation of the Master Mix Source Plate for Post-CaptureOnBeadPCR_XT_Illumina_v2.0.pro

SureSelect^{XT2} Reagent	Position on Source Plate	Volume of Reagents 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
SureSelect Herculase II Master Mix	Column 4 (A4-H4)	37.5 µL	62.5 µL	87.5 µL	112.5 µL	162.5 µL	325 µL
XT2 Primer Mix		1.5 µL	2.5 µL	3.5 µL	4.5 µL	6.5 µL	13.0 µL

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

7 Vortex the plate for 5 seconds then 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.

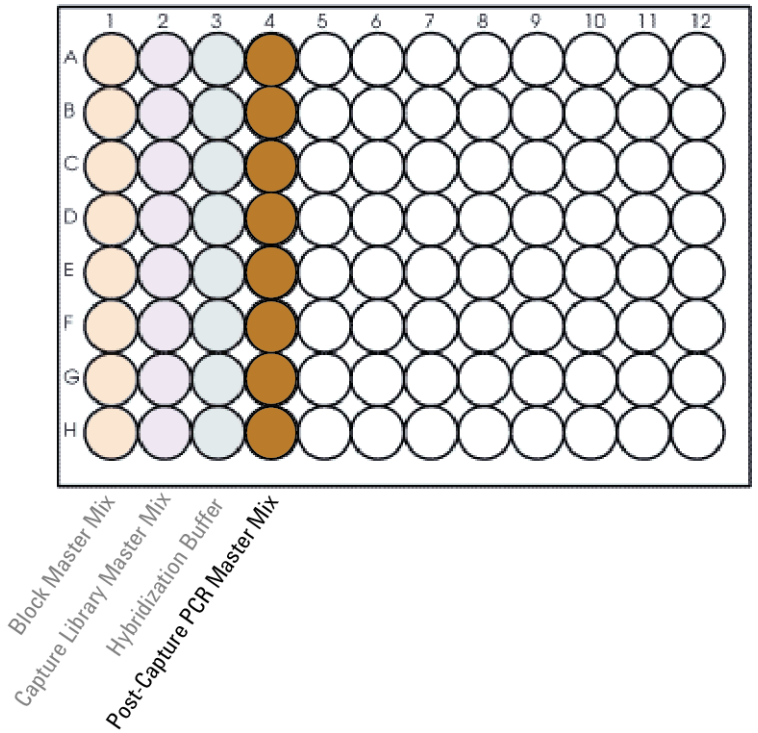


Figure 13 Configuration of the master mix source plate for Post-CaptureOnBeadPCR_XT_Illumina_v2.0.pro. Columns 1-3 may have been used to dispense master mixes for the Hybridization_MMCol_v2.0.pro protocol, or may be empty.

Load the Agilent NGS Workstation

8 Load the Labware MiniHub according to Table 53, using the plate orientations shown in Figure 6.

NOTE

Load a new tip box in Cassete 1, Shelf 1 for the Post-CaptureOnBeadPCR_XT_Illumina_v2.0.pro protocol. Do not retain a partially-filled tip box from previous runs.

Table 53 Initial MiniHub configuration for Post-CaptureOnBeadPCR_XT_Illumina_v2.0.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

9 Load the BenchCel Microplate Handling Workstation according to Table 54.

Table 54 Initial BenchCel configuration for Post-CaptureOnBeadPCR_XT_Illumina_v2.0.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	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

10 Load the Bravo deck according to Table 55.

Table 55 Initial Bravo deck configuration for Post-CaptureOnBeadPCR_XT_Illumina_v2.0.pro

Location	Content
5	Eppendorf twin.tec plate (unsealed) containing captured, bead-bound DNA samples
6	Empty PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
9	Master mix plate (unsealed) containing PCR Master Mix in Column 4 seated in silver insert

Run VWorks protocol Post-CaptureOnBeadPCR_XT_Illumina_v2.0.pro

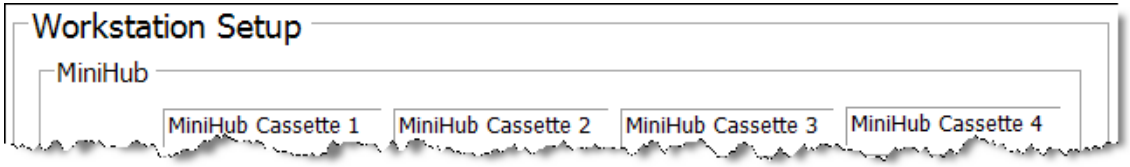
11 On the SureSelect setup form, under **Select Protocol to Run**, select **Post-CaptureOnBeadPCR_XT_Illumina_v2.0.pro.**

12 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate that was loaded on Bravo deck position 6.

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**.

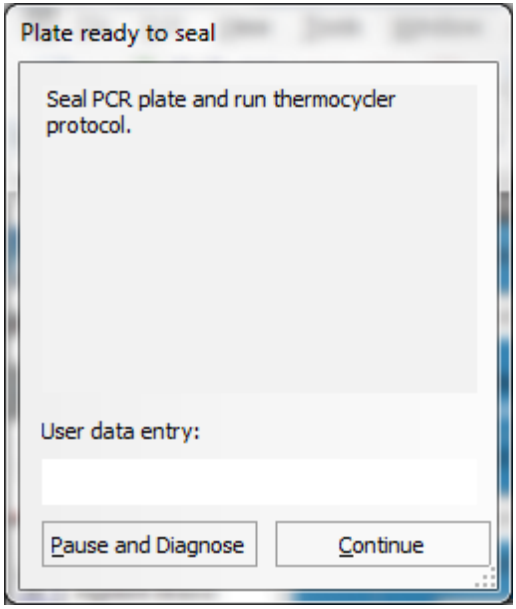
15 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

Running the Post-CaptureOnBeadPCR_XT_Illumina_v2.0.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 captured DNA samples, which may be stored for future use at –20°C, is located at position 5 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 56 using the cycle number specified in Table 57.

The volume of each PCR amplification reaction is 50 µL.

Table 56 Post-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8-14 see Table 57	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

Table 57 Recommended cycle number based on Capture Library size

Size of Capture Library	Cycles
<0.5 Mb	12 to 14 cycles
0.5 to 1.49 Mb	9 to 11 cycles
> 1.5 Mb (including All Exon and Exome libraries)	8 to 10 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 captured libraries using AMPure XP beads

In this step, the Agilent NGS Workstation transfers AMPure XP beads to the amplified captured DNA and then collects and washes the bead-bound enriched DNA amplicons.

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. Let the AMPure XP beads come to room temperature for at least 30 minutes.
4. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. *Do not freeze*.
5. 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.
6. Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water.
7. Prepare a separate Thermo Scientific reservoir containing 45 mL of freshly-prepared 70% ethanol.

8 Load the Labware MiniHub according to Table 58, using the plate orientations shown in Figure 6.

Table 58 Initial MiniHub configuration for DNA cleanup using SPRI_XT_Illumina_v2.0.pro:Post-CaptureOnBeadPCR Cleanup

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 twin.tec Plate	Empty	Empty
Shelf 2	Empty	Nuclease-free water reservoir from step 6	AMPure XP beads in Nunc DeepWell plate from step 5	Empty
Shelf 1 (Bottom)	Empty	70% ethanol reservoir from step 7	Empty	Empty tip box

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

Table 59 Initial BenchCel configuration for DNA cleanup using SPRI_XT_Illumina_v2.0.pro:Post-CaptureOnBeadPCR Cleanup

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

10 Load the Bravo deck according to Table 60.

Table 60 Initial Bravo deck configuration for DNA cleanup using SPRI_XT_Illumina_v2.0.pro:Post-CaptureOnBeadPCR Cleanup

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

Run VWorks protocol SPRI_XT_Illumina_v2.0.pro:Post-CaptureOnBeadPCR Cleanup

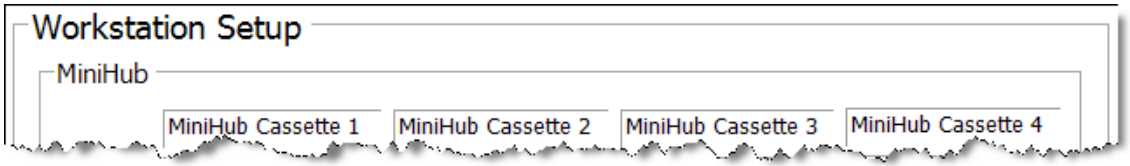
11 On the SureSelect setup form, under **Select Protocol to Run**, select **SPRI_XT_Illumina_v2.0.pro:Post-CaptureOnBeadPCR Cleanup.**

12 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate that was loaded on Bravo deck position 9.

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**.

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 quantity and quality of the amplified captured library pools

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

1 Set up the 2100 Bioanalyzer as instructed in the High Sensitivity DNA Assay kit guide.

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

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

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

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

NOTE Dilute 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 an average DNA amplicon size of approximately 250 to 300 bp. A sample electropherogram is shown in Figure 14.

7 Determine the concentration of each amplified captured library pool by integration under the peak in the electropherogram.

If the yield is too low or non-specific peaks are observed in the electropherogram, repeat the PCR with more or fewer cycles. The goal is to minimize cycles, while you produce enough library for application to the flow cell.

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.

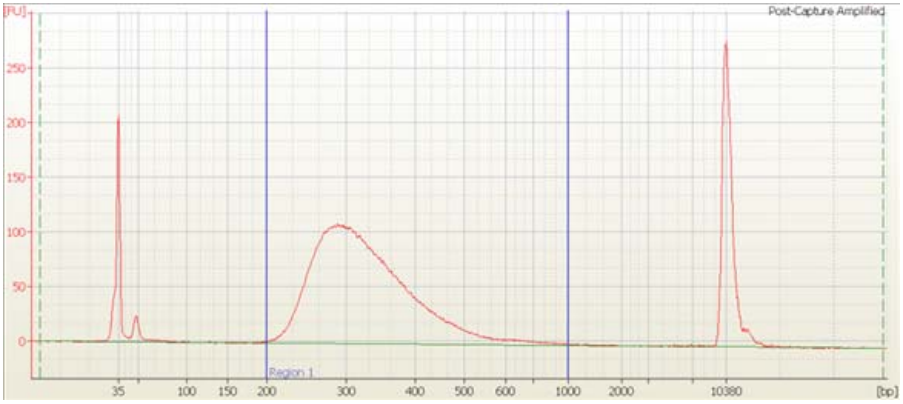


Figure 14 Analysis of amplified captured DNA using the 2100 Bioanalyzer and the High Sensitivity DNA Assay.

Option 2: Analysis using the Agilent 2200 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 amplified captured DNA. For more information to do this step, see the *Agilent 2200 TapeStation User Manual*.

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 *Agilent 2200 TapeStation User Manual*. Use 2 µL of each 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 2200 TapeStation as instructed in the *Agilent 2200 TapeStation User Manual*. Start the run.

5 Verify that the electropherogram shows an average DNA amplicon size of 250 to 300 bp. A sample electropherogram is shown in Figure 15.

6 Determine the concentration of each amplified captured library pool by integration under the peak in the electropherogram.

If the yield is too low or non-specific peaks are observed in the electropherogram, repeat the PCR with more or fewer cycles. The goal is to minimize cycles, while you produce enough library for application to the flow cell.

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.

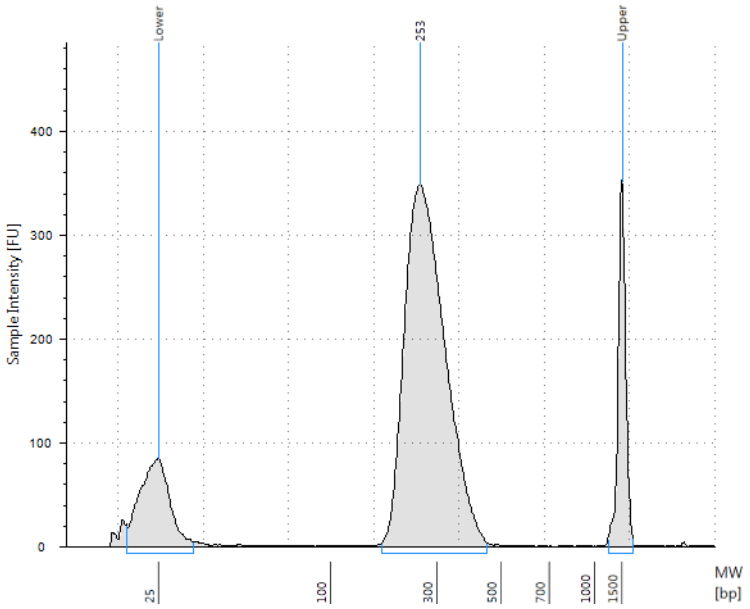


Figure 15 Analysis of amplified captured DNA using the 2200 TapeStation.

Step 4. Prepare samples for multiplexed sequencing

The final SureSelect^{XT2}-enriched samples contain pools of either 8 or 16 indexed libraries, based on the Capture Library used and resulting pre-capture pooling strategy. When appropriate for your sequencing platform, the 8-plex or 16-plex samples may be further multiplexed by post-capture pooling.

Determine whether to do post-capture pooling by calculating the number of indexes that can be combined per lane, according to the capacity of your platform, together with the amount of sequencing required to achieve the needed coverage for your specific Capture Library for each indexed sample.

If doing post-capture pooling, use the guidelines provided in "Step 6. Optional: Pool captured libraries for sequencing" on page 124. Prior to post-capture pooling, the DNA concentration of each sample may be accurately determined as described in "Step 5. Optional: Quantify captured library pools by QPCR" on page 123.

If samples will not be further combined in post-capture pools, proceed to cluster amplification using the Illumina Paired-End Cluster Generation Kit. Refer to the manufacturer's instructions for this step.

The optimal seeding concentration for SureSelect^{XT2} target-enriched libraries is 6 to 8 pM on HiSeq or MiSeq instruments and 1.2 to 1.3 pM on the NextSeq platform. 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.

Sequencing run setup guidelines

Sequencing runs must be set up to perform an 8-bp index read. For complete 8-bp index sequence information, see the Reference chapter starting on page 127.

For the HiSeq 2500 and NextSeq 500 (v1) platforms, use the *Cycles* settings shown in Table 61. 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 61 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 62.

Table 62 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 the index sequence tables in the Reference chapter starting on page 127).

Step 5. Optional: Quantify captured library pools by QPCR

For accurate determination of the DNA concentration in each captured library pool, use the QPCR NGS Library Quantification Kit (for Illumina).

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

1. Prepare a standard curve using the quantification standard included in the kit, according to the instructions provided in the user guide.
2. Dilute each captured library pool 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.
3. Prepare the QPCR master mix with Illumina adaptor-specific PCR primers according to instructions provided in the kit.
4. Add an aliquot of the master mix to PCR tubes and add template.
5. 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.
6. Use the standard curve to determine the concentration of each unknown captured library pool, in nM.

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

NOTE

In most cases, the cycle numbers in Table 57 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 6. Optional: Pool captured libraries for sequencing

See page 121 for post-capture pooling considerations, based on your SureSelect or ClearSeq Capture Library size and sequencing design. Pooling instructions are provided below.

1 Combine the capture pools such that each index-tagged sample is present in equimolar amounts in the final sequencing sample pool. For each final pool, use the formula below to determine the amount of each capture pool to use.

$$\text{Volume of capture pool} = \frac{V(f) \times C(f)}{\# \times C(i)} \quad \text{where}$$

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

C(f) is the desired final concentration of all the DNA in the pool

is the number of capture pool samples to be combined, and

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

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.

Table 63 shows an example of the amount of 2 capture pool samples (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM final DNA concentration.

Table 63 Example of capture pool volume calculations for a 20-µL final sequencing sample pool containing 10 nM DNA

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	20 µL	20 nM	10 nM	6	5.0
Sample 2	20 µL	15 nM	10 nM	6	6.7
Low TE					8.3

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

Proceed to cluster amplification using the Illumina Paired-End Cluster Generation Kit; refer to the manufacturer's instructions for this step. The optimal seeding concentration for cluster amplification from SureSelect^{XT2} DNA libraries is approximately 6 to 8 pM.

NOTE

The optimal seeding concentration may vary, depending on the method used for library quantification and fragment size distribution.

See page 121 for sequencing run setup guidelines for SureSelect^{XT2} libraries.

6 Reference

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

CAUTION This chapter contains two sets of index sequence and kit content information. The first section covers kits with indexing primers supplied in a blue plate format in Library Prep Kit p/n 5500-0131 (typically received February, 2015 or later). The second section covers kits with indexing primers supplied in tube format, in p/n 5190-3936 and p/n 5190-3937 (typically received before February, 2015). **Verify that you are referencing the information appropriate for your kit version before you proceed.**

Reference Information for Kits with Revised Index Configuration (indexing primers in blue plate)

Use the reference information in this section if your kit includes Library Prep Kit p/n 5500-0131. If your kit does not include this component kit, see page 132 for kit content and indexing primer information.

Kit Contents

SureSelect^{XT2} Automation Reagent Kits contain the following components:

Table 64 SureSelect^{XT2} Automation Reagent Kit Content-Revised Index Configuration

Component Kits*	Storage Condition	G9661B (96 Samples)†	G9661C (480 Samples)‡
SureSelect XT2 Library Prep Kit, ILM	–20°C	5500-0131	5 x 5500-0131
SureSelect XT2 Pre-Capture Box 1	Room Temperature	5190-4076	5190-4077
SureSelect XT2 Pre-Capture Automation-ILM Module Box 2	–20°C	5190-4462	5190-4463

* See Table 65 through Table 67 for a list of reagents included in each component kit.

† Kits contain reagents to prepare indexed libraries from 96 gDNA samples and to enrich the samples in 6 or 12 hybridization and capture reactions (as appropriate for the specific Capture Library size and sample pooling format).

‡ Kits contain reagents to prepare indexed libraries from 480 gDNA samples and to enrich the samples in 30 or 60 hybridization and capture reactions (as appropriate for the specific Capture Library size and sample pooling format).

NOTE SureSelect reagents and Capture Libraries must be used within one year of receipt.

The contents of each of the component kits listed in Table 64 are described in the tables below.

Table 65 SureSelect XT2 Library Prep Kit, ILM Content-Revised Configuration

Kit Component	Format
SureSelect End Repair Enzyme Mix	bottle
SureSelect End Repair Nucleotide Mix*	tube with green cap
SureSelect dA-Tailing Master Mix	bottle
SureSelect Ligation Master Mix	tube with purple cap
SureSelect Herculase II Master Mix	bottle
XT2 Primer Mix	tube with clear cap
SureSelect Pre-Capture Indexed Adaptors†	Indexes A01 through H12, provided in blue 96-well plate‡

* May also be labeled as *SureSelect End Repair Oligo Mix*.

† See Table 69 on page 131 for index sequences.

‡ See Table 68 on page 130 for a plate map.

Table 66 SureSelect XT2 Pre-Capture Box 1 Content

Kit Component	Format
SureSelect XT2 Binding Buffer	bottle
SureSelect XT2 Wash 1	bottle
SureSelect XT2 Wash 2	bottle

Table 67 SureSelect XT2 Pre-Capture Automation-ILM Module Box 2 Content

Kit Component	96 Sample Kit	480 Sample Kit
SureSelect XT2 Blocking Mix	tube with blue cap	tube with blue cap
SureSelect XT2 Hybridization Buffer	tube with yellow cap	bottle
SureSelect RNase Block	tube with purple cap	tube with purple cap

Table 68 Plate map for indexed adaptors containing indexes A01 through H12 (blue plate in Library Prep kit p/n 5500-0131)

	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^{XT2} Indexes A01 to H12

Each index is 8 nt in length. See page 121 for sequencing run setup information using 8-bp indexes.

Table 69 SureSelect^{XT2} 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

Reference Information for Kits with Original Index Configuration (indexing primers in clear-capped tubes)

Use the reference information in this section if your kit includes Pre-Capture Indexes in tube format in p/n 5190-3936 and p/n 5190-3937. If your kit does not include these component kits, see page 128 for kit content and indexing primer information.

Kit Contents

SureSelect^{XT2} Automation Reagent Kits contain the following component kits:

Table 70 SureSelect^{XT2} Automation Reagent Kit Content-Original Index Configuration

Component Kits*	Storage Condition	G9661B (96 Samples)†	G9661C (480 Samples)‡
SureSelect XT2 Library Prep Kit, ILM	–20°C	5500-0103	5 x 5500-0103
SureSelect XT2 Pre-Capture Indexes, ILM**	–20°C	5190-3936 and 5190-3937	5 x 5190-3936 and 5 x 5190-3937
SureSelect XT2 Pre-Capture Box 1	Room Temperature	5190-4076	5190-4077
SureSelect XT2 Pre-Capture Automation-ILM Module Box 2	–20°C	5190-4462	5190-4463

* See Table 71 through Table 74 for a list of reagents included in each component kit.

† Kits contain reagents to prepare indexed libraries from 96 gDNA samples and to enrich the samples in 6 or 12 hybridization and capture reactions (as appropriate for the specific Capture Library size and sample pooling format).

‡ Kits contain reagents to prepare indexed libraries from 480 gDNA samples and to enrich the samples in 30 or 60 hybridization and capture reactions (as appropriate for the specific Capture Library size and sample pooling format).

** See Table 75 on page 134 through Table 80 on page 139 for index sequence information.

NOTE SureSelect reagents and Capture Libraries must be used within one year of receipt.

The contents of each of the component kits listed in Table 70 are described in the tables below.

Table 71 SureSelect XT2 Library Prep Kit, ILM Content-Original Index Configuration

Kit Component	Format
SureSelect End-Repair Master Mix	bottle
SureSelect dA-Tailing Master Mix	bottle
SureSelect Ligation Master Mix	tube with purple cap
SureSelect Herculase II Master Mix	bottle
XT2 Primer Mix	tube with clear cap

Table 72 SureSelect XT2 Pre-Capture Indexes Content-Original Index Configuration

Kit Component	Format
Indexes 1-48	48 clear-capped tubes, supplied in component kit 5190-3936
Indexes 49-96	48 clear-capped tubes, supplied in component kit 5190-3937

Table 73 SureSelect XT2 Pre-Capture Box 1 Content

Kit Component	Format
SureSelect XT2 Binding Buffer	bottle
SureSelect XT2 Wash 1	bottle
SureSelect XT2 Wash 2	bottle

Table 74 SureSelect XT2 Pre-Capture Automation-ILM Module Box 2 Content

Kit Component	96 Sample Kit	480 Sample Kit
SureSelect XT2 Blocking Mix	tube with blue cap	tube with blue cap
SureSelect XT2 Hybridization Buffer	tube with yellow cap	bottle
SureSelect RNase Block	tube with purple cap	tube with purple cap

Nucleotide Sequences of SureSelect Pre-Capture Indexes-Original Index Configuration

The nucleotide sequence of each SureSelect^{XT2} Pre-Capture Index provided with the original kit configuration is provided in the tables below.

Refer to the sequence information below only if your kit includes p/n 5190-3936 and p/n 5190-3937, with indexing primers provided in 96 individual clear-capped tubes.

Each index is 8 nt in length. Each index is 8 nt in length. See page 121 for sequencing run setup information using 8-bp indexes.

Table 75 SureSelect^{XT2} Pre-capture Indexes 1-16

Index Number	Sequence
1	AACGTGAT
2	AAACATCG
3	ATGCCTAA
4	AGTGGTCA
5	ACCACTGT
6	ACATTGGC
7	CAGATCTG
8	CATCAAGT
9	CGCTGATC
10	ACAAGCTA
11	CTGTAGCC
12	AGTACAAG
13	AACAACCA
14	AACCGAGA
15	AACGCTTA
16	AAGACGGA

Table 76 SureSelect^{XT2} Pre-capture Indexes 17-32

Index Number	Sequence
17	AAGGTACA
18	ACACAGAA
19	ACAGCAGA
20	ACCTCCAA
21	ACGCTCGA
22	ACGTATCA
23	ACTATGCA
24	AGAGTCAA
25	AGATCGCA
26	AGCAGGAA
27	AGTCACTA
28	ATCCTGTA
29	ATTGAGGA
30	CAACCACA
31	CAAGACTA
32	CAATGGAA

Table 77 SureSelect^{XT2} Pre-capture Indexes 33-48

Index Number	Sequence
33	CACTTCGA
34	CAGCGTTA
35	CATACCAA
36	CCAGTTCA
37	CCGAAGTA
38	CCGTGAGA
39	CCTCCTGA
40	CGAACTTA
41	CGACTGGA
42	CGCATACA
43	CTCAATGA
44	CTGAGCCA
45	CTGGCATA
46	GAATCTGA
47	GACTAGTA
48	GAGCTGAA

Table 78 SureSelect^{XT2} Pre-capture Indexes 49-64

Index Number	Sequence
49	GATAGACA
50	GCCACATA
51	GCGAGTAA
52	GCTAACGA
53	GCTCGGTA
54	GGAGAACA
55	GGTGCGAA
56	GTACGCAA
57	GTCGTAGA
58	GTCTGTCA
59	GTGTTCTA
60	TAGGATGA
61	TATCAGCA
62	TCCGTCTA
63	TCTTCACA
64	TGAAGAGA

Table 79 SureSelect^{XT2} Pre-capture Indexes 65-80

Index Number	Sequence
65	TGGAACAA
66	TGGCTTCA
67	TGGTGGTA
68	TTCACGCA
69	AACTCACC
70	AAGAGATC
71	AAGGACAC
72	AATCCGTC
73	AATGTTGC
74	ACACGACC
75	ACAGATTC
76	AGATGTAC
77	AGCACCTC
78	AGCCATGC
79	AGGCTAAC
80	ATAGCGAC

Table 80 SureSelect^{XT2} Pre-capture Indexes 81-96

Index Number	Sequence
81	ATCATTCC
82	ATTGGCTC
83	CAAGGAGC
84	CACCTTAC
85	CCATCCTC
86	CCGACAAC
87	CCTAATCC
88	CCTCTATC
89	CGACACAC
90	CGGATTGC
91	CTAAGGTC
92	GAACAGGC
93	GACAGTGC
94	GAGTTAGC
95	GATGAATC
96	GCCAAGAC

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In This Book

This guide contains information to run the SureSelect^{XT2} Automated Library Prep and Capture System protocol using the automation protocols provided with the Agilent NGS Workstation Option B.

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