SureSelect^{QXT} Automated Whole Genome Library Prep for Illumina Multiplexed Sequencing

Featuring Transposase-Based Library Prep Technology

Automated using Agilent NGS Workstation Option B

Protocol

Version E0, January 2021

SureSelect platform manufactured with Agilent SurePrint Technology

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

Agilent Technologies

Notices

Manual Part Number

G9682-90010

Edition

Version E0, January 2021

Printed in USA

Agilent Technologies, Inc.
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Santa Clara, CA 95051 USA

Acknowledgment

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In this Guide...

This guide describes an automated protocol for whole genome Illumina paired-end multiplexed library preparation using the SureSelect^{QXT} Library Prep system. Sample processing steps are automated using Agilent's NGS Workstation.

What's New in Version E0

- Updates to thermal cycler and plasticware recommendations (see Table 2 on page 13 and see *Caution* on page 31)
- Updates to ordering information for AMPure XP Kits and 1X Low TE Buffer (see Table 1 on page 12) and for Qubit Fluorometer (see Table 2 on page 13)
- Minor updates to Agilent Bioanalyzer assay use instructions and reference document links (see page 57)
- Updates to downstream sequencing support information including sequencing kit selection and seeding concentration updates (see Table 27 on page 61) and support for the NovaSeq platform (see page 61 through page 66 and see page 72)
- Updates to instructions for adaptor trimming using Agilent's AGeNT Trimmer utility and removal of SureCall adaptor trimming information (see page 66 to page 67)
- Removal of reference information for expired SureSelect^{QXT} Reagent Kit p/n G9682B, replaced by G9684B in 2018 (see Table 1 on page 12 and see Table 39 through Table 41 on page 70 for current Reagent Kit information)
- Updates to Technical Support contact information (see page 2)
- Updates to *Notice to Purchaser* (see page 2)

What's New in Version D0

- Support for replacement of SureSelect^{QXT} Reagent Kit p/n G9682B with p/n G9684B for use with Illumina's HiSeq and MiSeq platforms (see Table 1 on page 12, Table 39 on page 70, and Table 41 on page 70)
- Support for VWorks software version 13.1.0.1366 and Agilent NGS Workstation Option B p/n G5574AA (see Table 2 on page 13)

- Support for use of Illumina's HiSeq 3000 and HiSeq 4000 platforms for downstream sequencing steps (see page 61 through page 63 and Table 40 on page 72)
- Updates to sequencing kit selection and seeding concentration guidelines (see page 61)
- Updates to custom sequencing primer dilution instructions (See page 63 to page 64)
- Update to sequencing run setup recommendations (see"HiSeq or NextSeq 500 platform sequencing run setup and adaptor trimming guidelines" on page 66)
- Updates to dual index multiplexing guidelines (see Table 45 on page 73)
- Updates to Agilent 2100 Bioanalyzer system ordering information (see page 14)
- Updates to reference information for Agilent NGS Workstation component user guides (see Table 3 on page 16)
- Updates to product guarantee and support statement (see *Note* on page 9)
- Updates to supplier name for materials purchased from Thermo Fisher Scientific (see Table 1 on page 12 and Table 2 on page 13)
- Updates to Technical Support contact information (see page 2)

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

Procedural Notes

- The SureSelect^{QXT} system requires high-quality DNA samples for optimal performance. Use best practices for verifying DNA sample quality before initiating the workflow. For best practice, store diluted DNA solutions at 4°C to avoid repeated freeze-thaw cycles, which may compromise DNA quality.
- Performance of the SureSelect^{QXT} library preparation protocol is very sensitive to variations in amounts of DNA sample and other reaction components. It is important to quantify and dilute DNA samples as described on page 34. Carefully measure volumes for all reaction components, and combine components as described in this instruction manual. Use best-practices for liquid handling, including regular pipette calibration, to ensure precise volume measurement.
- Use care in handling the SureSelect QXT Enzyme Mix. After removing the vial from storage at –20°C, keep on ice or in a cold block while in use. Return the vial to storage at –20°C promptly after use.
- Use best-practices to prevent PCR product contamination of samples throughout the workflow:

  1. Assign separate pre-PCR and post-PCR pipettors, supplies, and reagents. In particular, never use materials designated to post-PCR segments for the pre-PCR segments of the workflow. For the pre-PCR workflow steps, always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
  2. Maintain clean work areas. Clean pre-PCR surfaces that pose the highest risk of contamination daily using a 10% bleach solution.
  3. Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.

- Possible stopping points, where samples may be stored at –20°C, are marked in the protocol. Do not subject the samples to multiple freeze/thaw cycles.
- 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.
- In general, follow Biosafety Level 1 (BSL1) safety rules.

Safety Notes

CAUTION

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

Required Reagents

Table 1 Required Reagents for SureSelect^{QXT} Whole Genome Library Prep

Description	Vendor and part number
SureSelect^{QXT} Library Prep Kit, 96 Samples (for Illumina HiSeq, MiSeq, and NextSeq platforms)	Agilent p/n G9684B
AMPure XP Kit 5 mL 60 mL 450 mL	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
1X Low TE Buffer (10 mM Tris-HCl, pH 7.5-8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Qubit dsDNA HS Assay Kit *or* Qubit dsDNA BR Assay Kit 100 assays 500 assays	Thermo Fisher Scientific p/n Q32851 Thermo Fisher Scientific p/n Q32850 p/n Q32853
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

Required Equipment

Table 2 Required Equipment for SureSelect^{QXT} Whole Genome Library Prep

Description	Vendor and part number
Agilent NGS Workstation Option B Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5522A (VWorks software version 13.1.0.1366, 13.0.0.1360, or 11.3.0.1195) OR Agilent p/n G5574AA (VWorks software version 13.1.0.1366)
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Clear Peelable Seal plate seals (for use with the PlateLoc Thermal Plate Sealer)	Agilent p/n 16985-001
Thermal cycler and accessories	Various suppliers *Important*: Not all PCR plate types are supported for use in the VWorks automation protocols for the Agilent NGS Workstation. Select a thermal cycler that is compatible with one of the supported PCR plate types. See supported plate types in the listing below.
PCR plates compatible with the Agilent NGS Workstation and associated VWorks automation protocols	Only the following PCR plates are supported: • 96 ABI PCR half-skirted plates (MicroAmp Optical plates), Thermo Fisher Scientific p/n N8010560 • 96 Agilent semi-skirted PCR plate, Agilent p/n 401334 • 96 Eppendorf Twin.tec half-skirted PCR plates, Eppendorf p/n 951020303 • 96 Eppendorf Twin.tec PCR plates (full-skirted), Eppendorf p/n 951020401
Eppendorf twin.tec full-skirted 96-well PCR plates	Eppendorf p/n 951020401 or 951020619
Thermo Scientific Reservoirs	Thermo Fisher Scientific p/n 1064156
Nunc DeepWell Plates, sterile, 1.3-mL well volume	Thermo Fisher Scientific p/n 260251
Axygen 96 Deep Well Plate, 2 mL, Square Well (waste reservoirs; working volume 2.2 mL)	Axygen p/n P-2ML-SQ-C E & K Scientific p/n EK-2440
Nucleic acid surface decontamination wipes	DNA Away Surface Decontaminant Wipes, Thermo Fisher Scientific p/n 7008, or equivalent

Table 2 Required Equipment for SureSelect^{QXT} Whole Genome Library Prep

Description	Vendor and part number
DNA Analysis Platform and Consumables	
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
Agilent DNA 1000 Kit	Agilent p/n 5067-1504
Agilent High Sensitivity DNA Kit	Agilent p/n 5067-4626
Qubit Fluorometer	Thermo Fisher Scientific p/n Q33238
Qubit Assay Tubes	Thermo Fisher Scientific p/n Q32856
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Plate or strip tube centrifuge	Labnet International MPS1000 Mini Plate Spinner p/n C1000 (requires adapter, p/n C1000-ADAPT, for use with strip tubes) or equivalent
Multichannel pipette	Rainin Pipet-Lite Multi Pipette or equivalent
P10, P20, P200 and P1000 pipettes	Rainin Pipet-Lite Pipettes or equivalent
Vortex mixer	General laboratory supplier
Ice bucket	General laboratory supplier
Powder-free gloves	General laboratory supplier
Sterile, nuclease-free aerosol barrier pipette tips	General laboratory supplier

2
Using the Agilent NGS Workstation for SureSelect QXT Whole Genome Library Prep

This chapter contains an orientation to the Agilent NGS Workstation, an overview of the SureSelect^{QXT} whole genome library preparation protocol, and considerations for designing SureSelect^{QXT} experiments for automated processing using the Agilent NGS Workstation.

About the Agilent NGS Workstation

CAUTION Before you begin, make sure that you have read and understand operating, maintenance and safety instructions for using the Bravo platform and additional devices included with the workstation. Refer to the user guides listed in Table 3.

Review the user guides listed in Table 3 (available at Agilent.com) to become familiar with the general features and operation of the Agilent NGS Workstation Option B components. Instructions for using the Bravo platform and other workstation components for the SureSelect^{QXT} Whole Genome workflow are detailed in this user guide.

Table 3 Agilent NGS Workstation components User Guide reference information

Device	User Guide part number
Bravo Platform	G5562-90000
VWorks Software	G5415-90068 (VWorks versions 13.1.0.1366 and 13.0.0.1360), or G5415-90063 (VWorks version 11.3.0.1195)
BenchCel Microplate Handler	G5400-90004
Labware MiniHub	G5471-90002
PlateLoc Thermal Microplate Sealer	G5402-90001

About the Bravo Platform

The Bravo platform is a versatile liquid handler with a nine plate-location platform deck, suitable for handling 96-well, 384-well, and 1536-well plates. The Bravo platform is controlled by the VWorks Automation Control software. Fitted with a choice of seven interchangeable fixed-tip or disposable-tip pipette heads, it accurately dispenses fluids from 0.1 µl to 250 µl.

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.

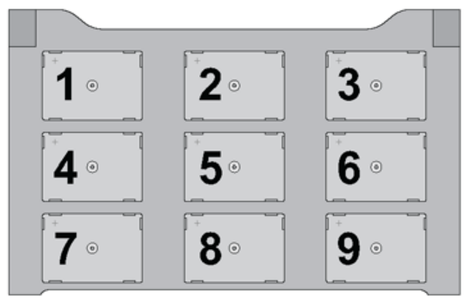


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

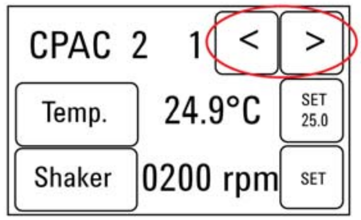
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

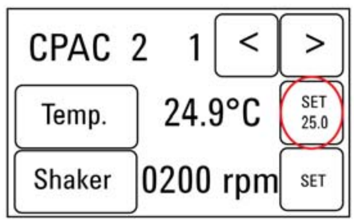
Table 4 Inheco Multi TEC Control touchscreen designations

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

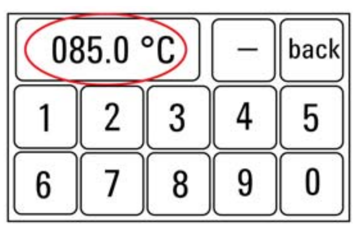
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



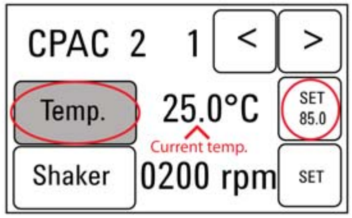
2 To set the temperature of the selected block, press the SET button.



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



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



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

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

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

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

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

3 Press the **START** button.

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

Using the Labware MiniHub

The protocols in the following sections include instructions for placing plates or reservoirs at specific Labware MiniHub positions. Use Figure 2 to familiarize yourself with the required orientations loading plates in the Labware MiniHub for use in SureSelect automation protocols.

For Thermo Scientific reservoirs, place the notched corner facing the center of the hub.

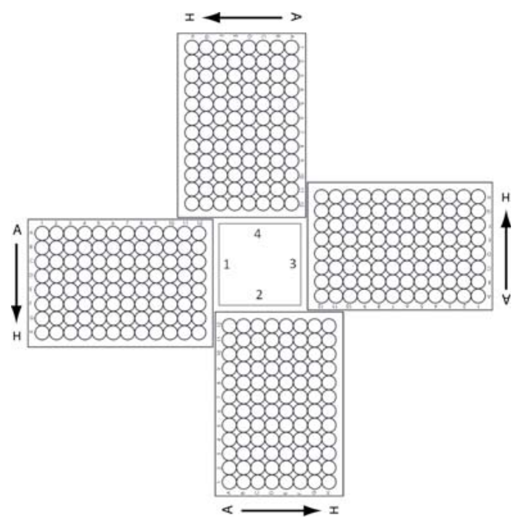


Figure 2 Agilent Labware MiniHub plate orientation.

VWorks Automation Control Software

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

NOTE

The instructions in this manual are compatible with VWorks software version 13.0.0.1360 or 11.3.0.1195, including SureSelect^{QXT} automation protocols version 1.0.

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

Logging in to the VWorks software

1 Double-click the VWorks icon or the SureSelectQXT_ILM_v1.0.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.

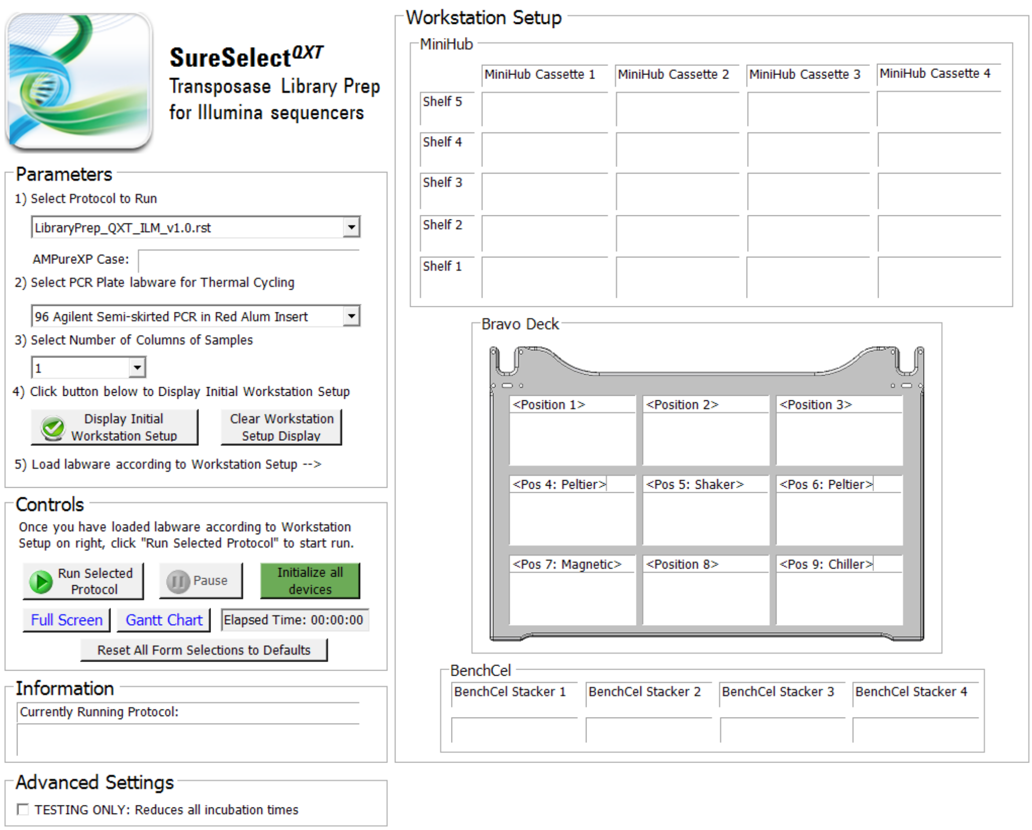
3 In the User Authentication dialog, type your VWorks user name and password, and click **OK**. (If no user account is set up, contact the administrator.)

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 SureSelectQXT_ILM_v1.0.VWForm to setup and start a run

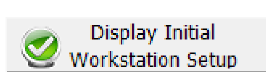
Use the VWorks form SureSelectQXT_ILM_v1.0.VWForm, shown below, to set up and start each SureSelect automation protocol or runset.



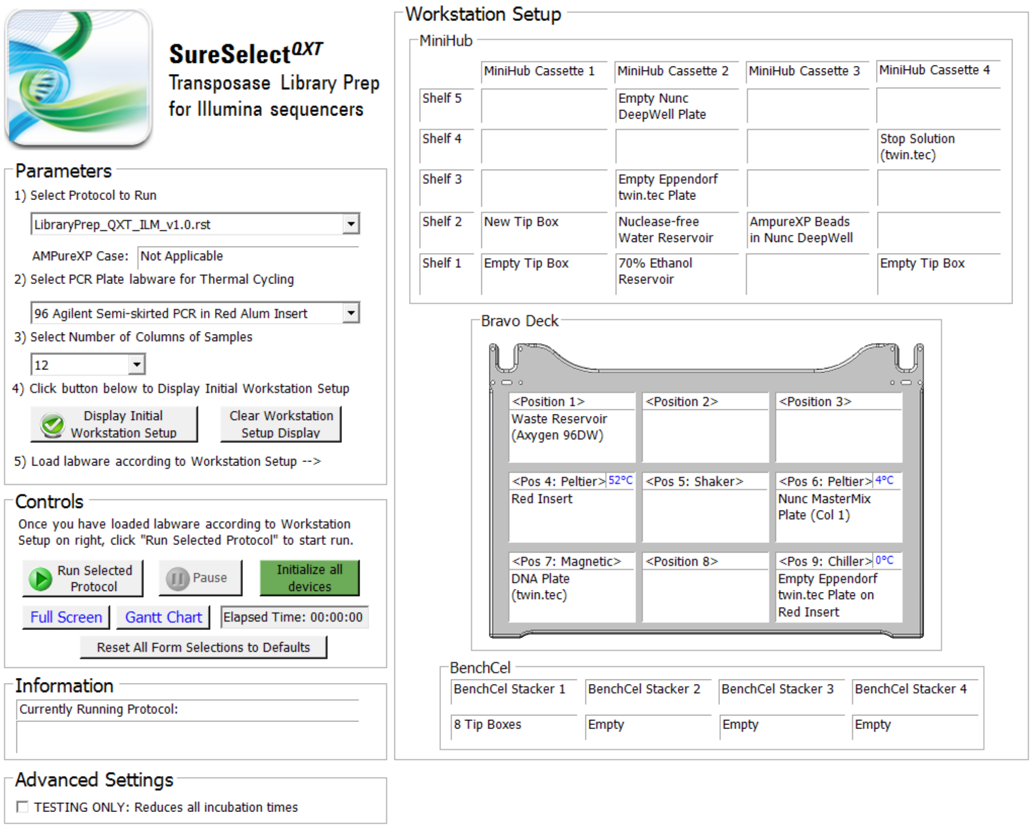
1 Open the form using the SureSelectQXT_ILM_v1.0.VWForm shortcut on your desktop.

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

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



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

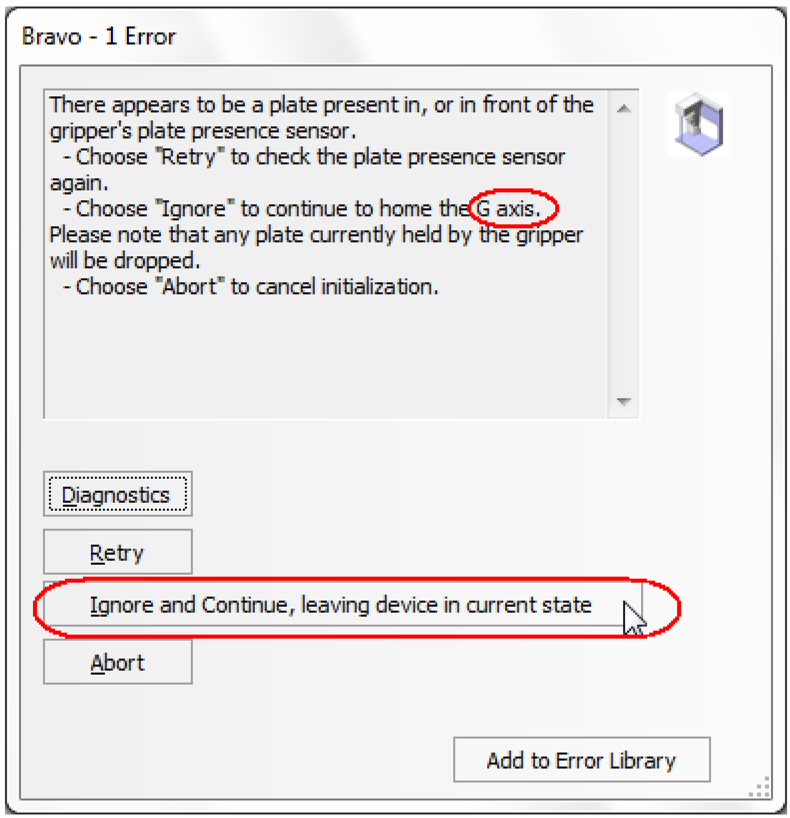


5 After verifying that the NGS Workstation has been set up correctly, click **Run Selected Protocol**.

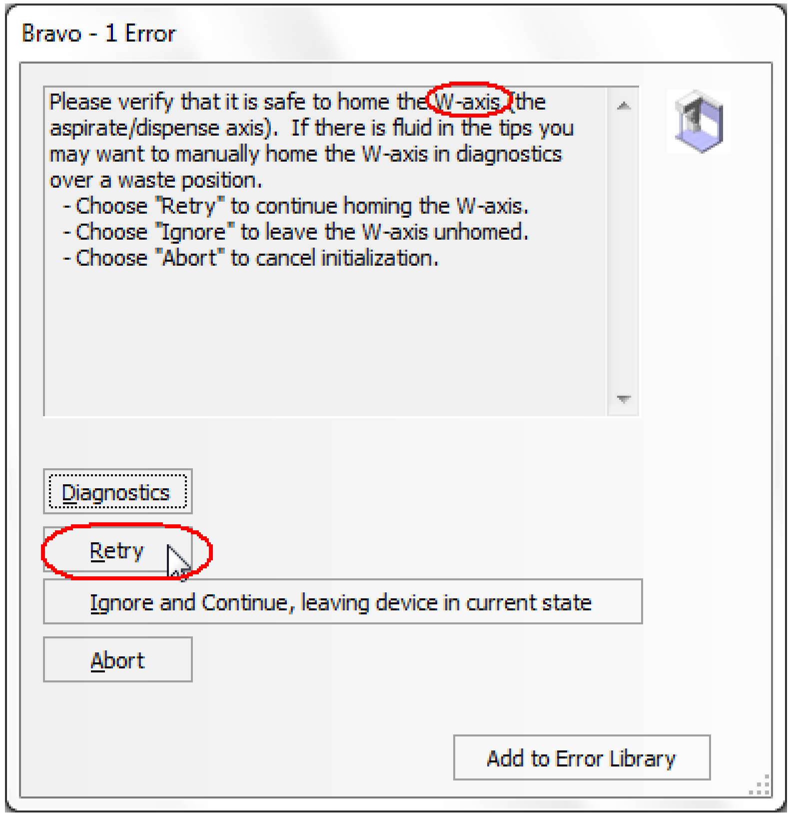
Error messages encountered at start of run

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

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



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



Verifying the Simulation setting

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

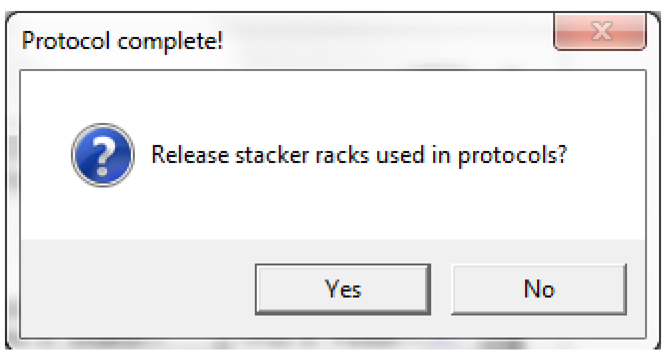
1 Verify that **Simulation is off** is displayed on the status indicator (accessible by clicking **View** > **Control Toolbar**).

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

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

Finishing a protocol or runset

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



Overview of the SureSelect^{QXT} Whole Genome Library Prep Procedure

Figure 3 summarizes the SureSelect^{QXT} whole genome library prep workflow for samples to be sequenced using the Illumina paired-read sequencing platform. Each sample to be sequenced requires an individual library preparation reaction. The samples are then tagged by PCR with dual index sequences. Depending on the experimental design, up to 96 samples can be pooled and sequenced in a single lane using the dual index tags that are provided with SureSelect^{QXT} Library Prep kits.

Table 5 summarizes how the VWorks protocols are integrated into the SureSelect^{QXT} workflow. See the Sample Preparation chapter for complete instructions for use of the VWorks protocols for sample processing.

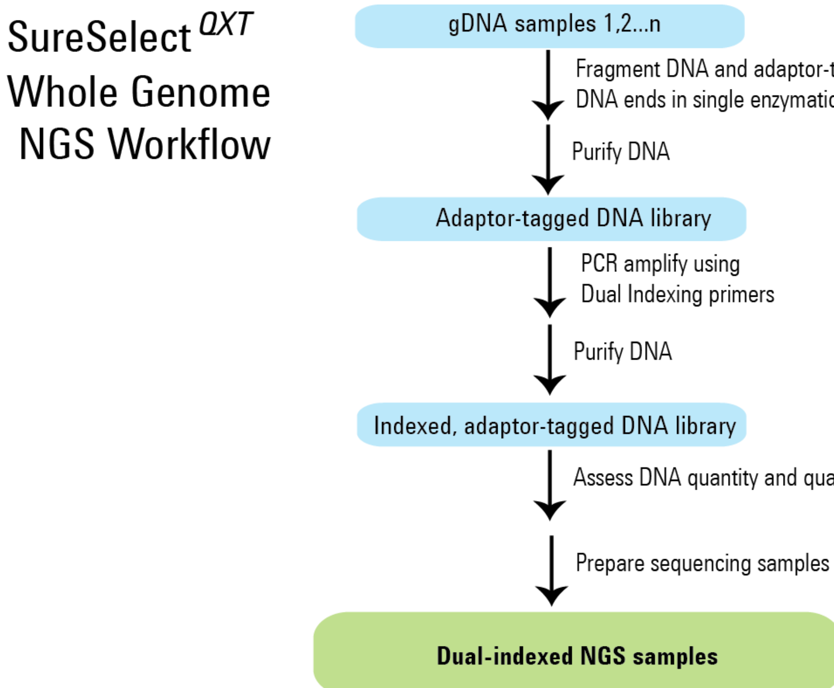


Figure 3 Overall sequencing sample preparation workflow.

Table 5 Overview of VWorks protocols and runsets used for SureSelect^{QXT} Whole Genome Library Prep

Workflow Step	VWorks Protocol Used for Agilent NGS Workstation automation
Prepare fragmented and adaptor-tagged DNA	LibraryPrep_QXT_ILM_v1.0.rst
Purify adaptor-tagged DNA using AMPure XP beads	
Amplify adaptor-tagged DNA and add index tags	WholeGenomePCR_QXT_ILM_v1.0.pro
Purify indexed DNA using AMPure XP beads	AMPureXP_QXT_ILM_v1.0.pro:Whole Genome PCR

Experimental Setup Considerations for Automated Runs

Agilent SureSelect 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 to be prepared for sequencing on the Illumina platform. Plan your experiments using complete columns of samples.

Table 6 Columns to Samples Equivalency

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

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

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

- The 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.
- Dual index assignments for the DNA samples can affect sample placement decisions at the beginning of the workflow. For example, all samples on the same row of the DNA sample plate must be assigned to the same P5 indexing primer during sample indexing (see Figure 3). It is important to review and understand the guidelines for assignment of dual indexing primers on page 45 while planning sample placement for the run to ensure that the indexing design is compatible with the initial DNA sample placement.

Considerations for Equipment Setup

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

PCR Plate Type Considerations

Automation protocols include 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 SureSelectQXT_ILM_v1.0.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 7.

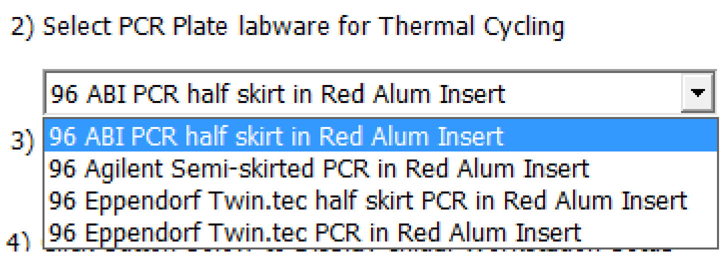


Table 7 Ordering information for supported PCR plates

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

CAUTION

The plates listed in Table 7 are compatible with the Agilent NGS Bravo and associated VWorks automation protocols, designed to support use of various thermal cyclers.

Do not use PCR plates that are not listed in Table 7 even if they are compatible with your chosen thermal cycler.

3
Sample Preparation

This section contains instructions for whole genome library preparation from gDNA specific to the Illumina paired-read sequencing platform and to automated processing using the Agilent NGS Workstation.

Step 1. Prepare the genomic DNA samples and Library Prep reagents

It is important to have all materials prepared in advance of use in the SureSelect^{QXT} automated Library Prep protocol. In this step, the gDNA is carefully quantified and dispensed into the sample plate. Additional reagents that require modification or temperature equilibration before use are also prepared in this step.

1. Remove the DMSO vial from the SureSelect QXT Library Prep Kit Box 2 in –20°C storage. Leave the DMSO vial at room temperature in preparation for use on page 45.
2. Prepare reagents for the purification protocols on page 37 and page 53.
   a. Transfer the AMPure XP beads to room temperature. The beads should be held at room temperature for at least 30 minutes before use. *Do not freeze the beads at any time.*
   b. Prepare 100 mL of fresh 70% ethanol for use in the purification steps. The 70% ethanol may be used for multiple steps done on the same day, when stored in a sealed container.
3. Obtain the bottle of SureSelect QXT Stop Solution from SureSelect QXT Library Prep Box 1 (stored at room temperature). Verify that the SureSelect QXT Stop Solution contains 25% ethanol, by referring to the container label and the instructions below.

   Before the first use of a fresh container, add 1.5 mL of ethanol to the provided bottle containing 4.5 mL of stop solution, for a final ethanol concentration of 25%. Seal the bottle then vortex well to mix. After adding the ethanol, be sure to mark the label for reference by later users.

   Keep the prepared 1X SureSelect QXT Stop Solution at room temperature, tightly sealed, until it is used on page 38.
4. Quantify and dilute gDNA samples using two serial fluorometric assays:
   a. Use the Qubit dsDNA BR Assay or Qubit dsDNA HS Assay to determine the initial concentration of each gDNA sample. Follow the manufacturer's instructions for the specific assay kit and the Qubit

instrument. This step is critical for successful preparation of input DNA at the required concentration to ensure optimal fragmentation.

b Dilute each gDNA sample with nuclease-free water to a final concentration of 100 ng/µL in a 1.5-mL LoBind tube.

c Carefully measure the DNA concentration of each of the 100 ng/µL dilutions using a second Qubit dsDNA BR or HS Assay.

d Adjust each gDNA sample with nuclease-free water to a final concentration of 10 ng/µL in a 1.5-mL LoBind tube.

5 Transfer 5 µL of the 10 ng/µL-DNA samples into the wells of a 96-well Eppendorf plate, column-wise, for processing on the Agilent NGS Workstation, in well order A1 to H1, then A2 to H2, ending with A12 to H12.

NOTE SureSelect Automated Library Prep runs may include 1, 2, 3, 4, 6, or 12 columns of the plate. See Experimental Setup Considerations for Automated Runs on page 29 for additional sample placement considerations.

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

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

Store the sample plate on ice until it is used on page 41.

Step 2. Fragment and adaptor-tag the genomic DNA samples

In this step, automation runset LibraryPrep_QXT_ILM_v1.0.rst is used to enzymatically fragment the gDNA and to add adaptors to ends of the fragments in a single reaction. After fragmentation and tagging, the Agilent NGS Workstation purifies the prepared DNA using AMPure XP beads.

This step uses the SureSelect^{QXT} Reagent Kit components listed in Table 8 in addition to reagents prepared for use on page 34 to page 35.

Table 8 Reagents for DNA fragmentation and adaptor-tagging

Kit Component	Storage Location	Where Used
SureSelect QXT Buffer	SureSelect QXT Library Prep Kit Box 2, –20°C	page 36
SureSelect QXT Enzyme Mix ILM	SureSelect QXT Library Prep Kit Box 2, –20°C	page 36

Prepare the workstation

1 Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2 Gently wipe down the Labware MiniHub, Bravo decks, and BenchCel with a DNA Away decontamination wipe.
3 Pre-set the temperature of Bravo deck position 4 to 52°C and position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. On the control touchscreen, Bravo deck position 4 corresponds to CPAC 2, position 1 and Bravo deck position 6 corresponds to CPAC 2, position 2.
4 Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
5 Place red PCR plate inserts at Bravo deck positions 4 and 9.

6 Load tip boxes for the run in the BenchCel Microplate Handling Workstation according to Table 9.

Table 9 Initial BenchCel configuration for LibraryPrep_QXT_ILM_v1.0.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	2 Tip boxes	Empty	Empty	Empty
4	3 Tip boxes	Empty	Empty	Empty
6	4 Tip boxes	Empty	Empty	Empty
12	8 Tip boxes	Empty	Empty	Empty

7 Load the workstation MiniHub with the empty plates and other labware components for the run, using the positions shown in the Workstation Setup region of the VWorks Form. Use the plate orientations shown in Figure 2 on page 20.

Prepare the purification reagents

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

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

10 Prepare a Nunc DeepWell source plate for the beads by adding 55 µL of homogeneous AMPure XP beads per well, for each well to be processed. Place the bead source plate on shelf 2 of cassette 3 of the workstation MiniHub.

11 Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water. Place the water reservoir on shelf 2 of cassette 2 of the workstation MiniHub.

12 Prepare a separate Thermo Scientific reservoir containing 45 mL of freshly-prepared 70% ethanol. Place the ethanol reservoir on shelf 1 of cassette 2 of the workstation MiniHub.

Prepare the Library Prep Master Mix and Stop Solution source plates

13 Prepare the Stop Solution source plate using an Eppendorf twin.tec full-skirted PCR plate. Add 35 µL of 1X SureSelect QXT Stop Solution per well, for each well to be processed. Place the source plate on shelf 4 of cassette 4 of the workstation MiniHub.

14 Before use, vortex the SureSelect QXT Buffer and SureSelect QXT Enzyme Mix ILM tubes vigorously at high speed.

These components are in liquid form when removed from –20°C storage and should be returned to –20°C storage promptly after use.

CAUTION

Minor variations in volumes of the solutions combined in step 15 below may result in DNA fragment size variation.

The SureSelect QXT Buffer and Enzyme Mix solutions are highly viscous. Thorough mixing of the reagents is critical for optimal performance.

15 Prepare the appropriate volume of Library Prep Master Mix, according to Table 10. Mix well by vortexing for 20 seconds and then keep on ice.

Table 10 Preparation of Library Prep Master Mix

SureSelect^{QXT} 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 QXT Buffer	17.0 µL	216.8 µL	361.3 µL	505.8 µL	650.3 µL	939.3 µL	1878.5 µL
Nuclease-free water	1.0 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
SureSelect QXT Enzyme Mix ILM	1.0 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
Total Volume	**19 µL**	**242.3 µL**	**403.8 µL**	**565.3 µL**	**726.8 µL**	**1049.8 µL**	**2099.5 µL**

16 Prepare the Library Prep master mix source plate using a Nunc DeepWell plate, containing the mixture from step 15. Add the volume indicated in Table 11 to all wells of column 1 of the Nunc DeepWell plate. Keep the master mix on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in Figure 4.

Table 11 Preparation of the Master Mix Source Plate for LibraryPrep_QXT_ILM_v1.0.rst

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Library Prep Master Mix	Column 1 (A1-H1)	27.9 µL	48.1 µL	68.3 µL	88.5 µL	128.8 µL	260.1 µL

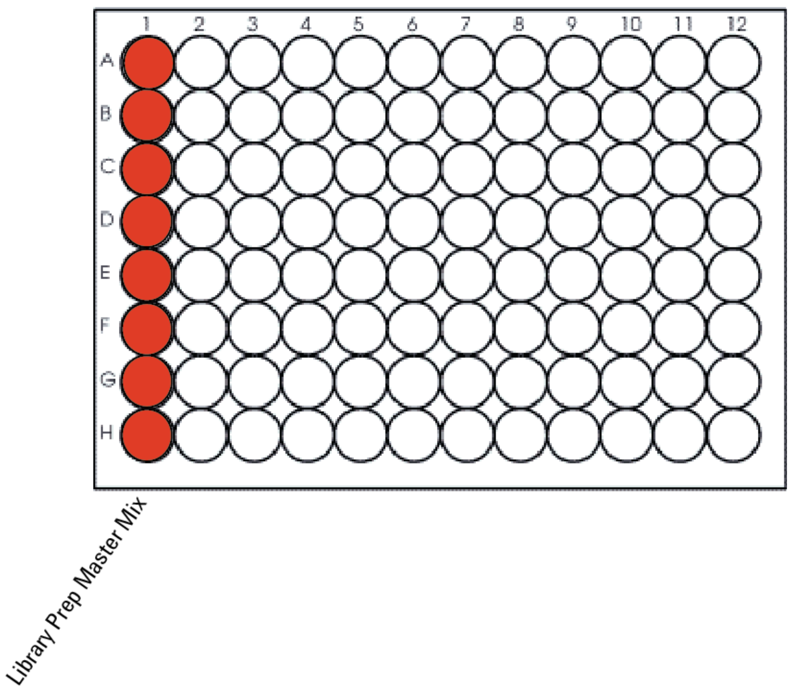


Figure 4 Configuration of the master mix source plate for LibraryPrep_QXT_ILM_v1.0.rst

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

18 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

19 Verify that the Labware MiniHub has been loaded as shown in Table 12.

Table 12 Initial MiniHub configuration for LibraryPrep_QXT_ILM_v1.0.rst

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty	Empty Nunc DeepWell plate	Empty	Empty
Shelf 4	Empty	Empty	Empty	Stop Solution source plate from step 13
Shelf 3	Empty	Empty Eppendorf plate	Empty	Empty
Shelf 2	New tip box	Nuclease-free water reservoir from step 11	AMPure XP beads in Nunc DeepWell plate from step 10	Empty
Shelf 1 (Bottom)	Empty tip box	70% ethanol reservoir from step 12	Empty	Empty tip box

20 Load the Bravo deck according to Table 13.

Table 13 Initial Bravo deck configuration for LibraryPrep_QXT_ILM_v1.0.rst

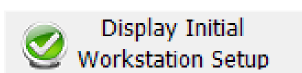
Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
4	Empty red insert
6	Library Prep Master Mix source plate (unsealed)
7	gDNA samples (5 µL of 10 ng/µL DNA per well) in Eppendorf plate (unsealed)
9	Empty Eppendorf plate on red insert

Run VWorks runset LibraryPrep_QXT_ILM_v1.0.rst

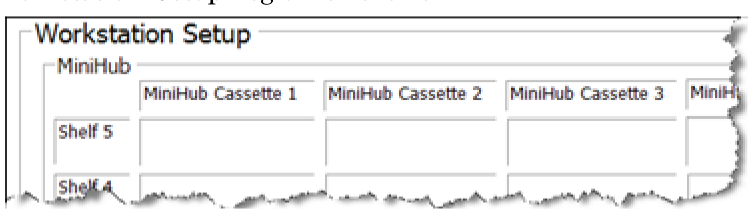
21 On the SureSelect setup form, under **Select Protocol to Run**, select **LibraryPrep_QXT_ILM_v1.0.rst.**

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

23 Click **Display Initial Workstation Setup**.

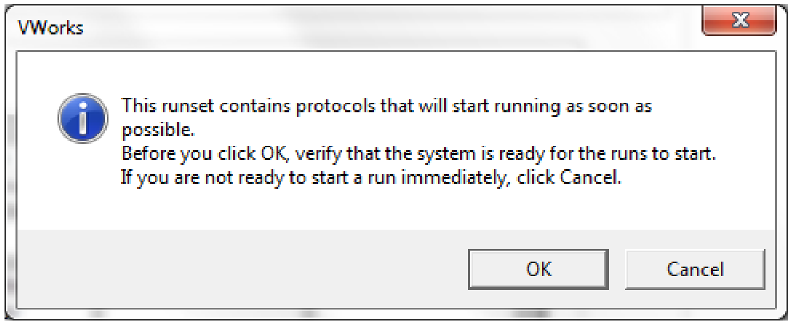


24 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

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



Running the LibraryPrep_QXT_ILM_v1.0.rst runset takes approximately 1 hour. Once complete, the purified, adaptor-ligated DNA samples are located in the Eppendorf plate at position 7 of the Bravo deck.

Step 3. Amplify adaptor-ligated libraries

In this step, the Agilent NGS Workstation completes the liquid handling steps for amplification and index tagging of the adaptor-ligated DNA samples using automation protocol WholeGenomePCR_QXT_ILM_v1.0.pro. Afterward, you transfer the PCR plate to a thermal cycler for amplification.

This step uses the SureSelect^{QXT} Reagent Kit components listed in Table 14.

Table 14 Reagents for whole genome library amplification

Kit Component	Storage Location	Where Used
Herculase II Fusion DNA Polymerase	SureSelect QXT Library Prep Kit Box 2, –20°C	page 45
Herculase II 5× Reaction Buffer	SureSelect QXT Library Prep Kit Box 2, –20°C	page 45
100 mM dNTP Mix (25 mM each dNTP)	SureSelect QXT Library Prep Kit Box 2, –20°C	page 45
SureSelect QXT P7 and P5 dual indexing primers	SureSelect QXT Library Prep Kit Box 2, –20°C	page 46
DMSO	Transferred to Room Temperature storage on page 34	page 45

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

Prepare the workstation

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

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

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

4 Load tip boxes for the run in the BenchCel Microplate Handling Workstation according to Table 15.

Table 15 Initial BenchCel configuration for WholeGenomePCR_QXT_ILM_v1.0.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

Prepare the PCR master mix and master mix source plate

5 Prepare the appropriate volume of PCR Master Mix, according to Table 16. Mix well using a vortex mixer and keep on ice.

Table 16 Preparation of PCR Master Mix

SureSelect^{QXT} Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	7.5 µL	95.6 µL	159.4 µL	223.1 µL	286.9 µL	414.4 µL	828.8 µL
Herculase II 5X Reaction Buffer	10.0 µL	127.5 µL	212.5 µL	297.5 µL	382.5 µL	552.5 µL	1105 µL
DMSO	2.5 µL	31.9 µL	53.1 µL	74.4 µL	95.6 µL	138.1 µL	276.3 µL
dNTP mix	0.5 µL	6.4 µL	10.6 µL	14.9 µL	19.1 µL	27.6 µL	55.3 µL
Herculase II Fusion DNA Polymerase	1.0 µL	12.8 µL	21.3 µL	29.8 µL	38.3 µL	55.3 µL	110.5 µL
Total Volume	**21.5 µL**	**274.1 µL**	**456.9 µL**	**639.6 µL**	**822.4 µL**	**1187.9 µL**	**2375.8 µL**

6 Using the same Nunc DeepWell master mix source plate that was used for the LibraryPrep_QXT_ILM_v1.0.rst runset, add the volume of PCR master mix indicated in Table 17 to all wells of column 2. Keep the source plate on ice until it is used on page 47.

Table 17 Preparation of the Master Mix Source Plate for WholeGenomePCR_QXT_ILM_v1.0.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
PCR Master Mix	Column 2 (A2-H2)	31.6 µL	54.4 µL	77.3 µL	100.1 µL	145.8 µL	294.3 µL

Assign and aliquot dual indexing primers

7 Determine the appropriate index assignments for each sample. See the Reference section for sequences of the index portion of the P7 (page 71) and P5 (page 72) indexing primers used to amplify the DNA libraries in this step.

Use the following guidelines for dual index assignments:

- Use a different indexing primer combination for each sample to be sequenced in the same lane.
- All samples on the same row of the prepared DNA library plate must be assigned to the same P5 indexing primer (P5 i13 through P5 i20). This design results from the automation protocol configuration in which the P5 indexing primer is dispensed from a single source plate column to all columns of the indexing PCR plate. Each row of samples may be assigned to the same or different P5 primers, depending on run size and multiplexing requirements. (See step 10, below, for P5 primer source plate setup details.)
- The automation protocol configuration allows for any of the provided P7 indexing primers (P7 i1 through P7 i12) to be assigned to any sample position of the prepared DNA library plate. (See step 8 and step 9 below, for P7 primer source plate setup details.)
- For sample multiplexing, Agilent recommends maximizing index diversity on both P7 and P5 primers as required for color balance. For example, when 8-plexing, use eight different P7 index primers with two P5 index primers. See Table 45 on page 73 for additional details.

8 Dilute each P7 indexing primer (P7 i1 through P7 i12) to be used in the run according to Table 18. The volumes below include the required excess.

Table 18 Preparation of P7 indexing primer dilutions

Reagent	Volume to Index 1 Sample	Volume to Index 8 Samples
Nuclease-free water	3.0 µL	25.5 µL
SureSelect QXT P7 dual indexing primer (P7 i1 to P7 i12)	2.0 µL	17 µL
Total Volume	**5.0 µL**	**42.5 µL**

9 In a fresh PCR plate, aliquot 5 µL of the appropriate P7 indexing primer dilution from Table 18 to the intended sample indexing well position(s).

Keep the plate on ice.

10 Using the Nunc DeepWell master mix source plate containing PCR Master Mix from step 6, add each P5 indexing primer (P5 i13 through P5 i20) to be used in the run to the appropriate well of column 2. Add the volume of P5 primer listed in Table 19 to each well of column 2, according to the number of sample columns in the run. Each well of column 2 can contain the same or different P5 indexing primers.

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

Table 19 Addition of P5 indexing primer to the DeepWell PCR source plate

Solution added to Source Plate	Position on Source Plate	Volume of Primer 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 QXT P5 dual indexing primer(s)*	Column 2 (A2-H2)	2.9 µL	5.1 µL	7.2 µL	9.3 µL	13.6 µL	27.4 µL

* Each well of column 2 may contain the same or different P5 indexing primer. Typical 12-column runs include all eight of the provided SureSelect QXT P5 dual indexing primers (P5 i13 through P5 i20), resulting in a different P5 primer assignment to each row of the PCR indexing plate.

NOTE If you are using a new DeepWell plate for the PCR source plate, leave column 1 empty and add the P5 Index(es) to column 2 of the new plate.

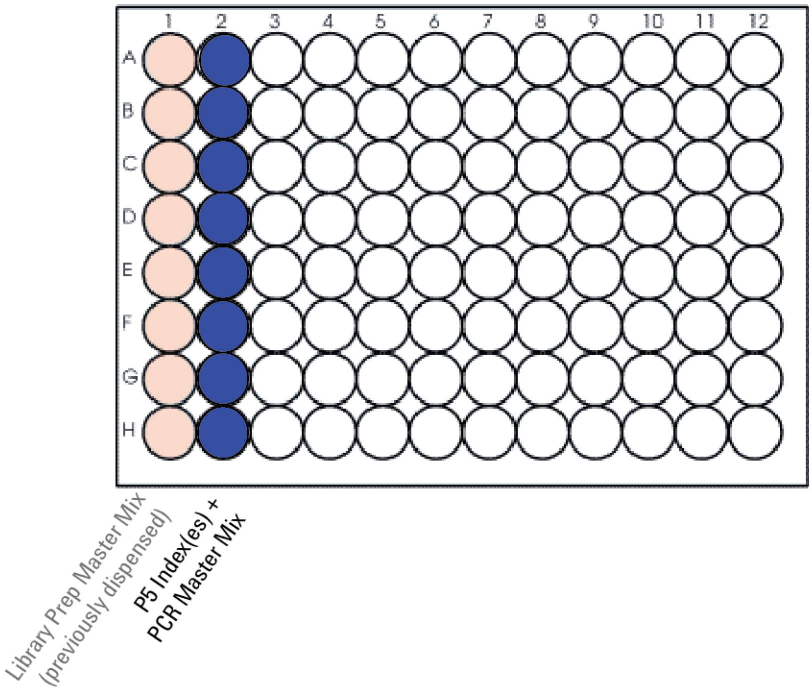


Figure 5 Configuration of the master mix source plate for WholeGenomePCR_QXT_ILM_v1.0.pro. Column 1 was used to dispense master mix during the previous protocol.

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

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

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

Load the Agilent NGS Workstation

13 Load the Labware MiniHub according to Table 20.

Table 20 Initial MiniHub configuration for WholeGenomePCR_QXT_ILM_v1.0.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	New tip box	Empty	Empty	Empty
Shelf 1 (Bottom)	Empty tip box	Empty	Empty	Empty tip box

14 Load the Bravo deck according to Table 21.

Table 21 Initial Bravo deck configuration for WholeGenomePCR_QXT_ILM_v1.0.pro

Location	Content
4	Adaptor-tagged DNA samples in Eppendorf plate
6	P7 indexing primers in PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
9	Master mix plate containing P5 indexing primers and PCR Master Mix in Column 2 (unsealed)

Run VWorks protocol WholeGenomePCR_QXT_ILM_v1.0.pro

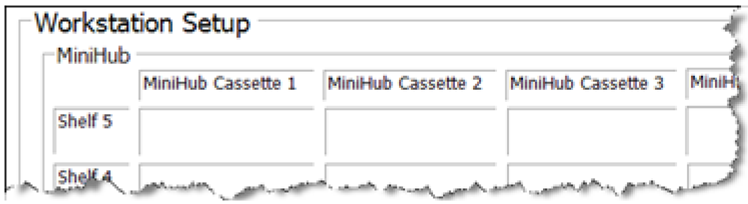
15 On the SureSelect setup form, under **Select Protocol to Run**, select **WholeGenomePCR_QXT_ILM_v1.0.pro.**

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

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

18 Click **Display Initial Workstation Setup**.

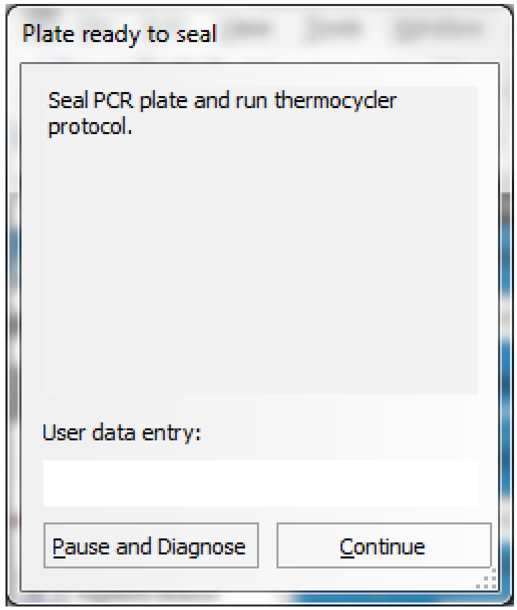
19 Verify that the NGS workstation has been set up as displayed in the Workstation Setup region of the form.



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

Running the WholeGenomePCR_QXT_ILM_v1.0.pro protocol takes approximately 15 minutes. Once complete, the PCR-ready samples, containing prepped DNA, dual indexing primers, and PCR master mix, are located in the PCR plate at position 6 of the Bravo deck.

21 When you see the following prompt, remove the PCR plate from position 6 of the Bravo deck and seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 3.0 seconds.



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

23 Transfer the PCR plate to a thermal cycler and run the PCR amplification program shown in Table 22.

Table 22 PCR cycling program

Segment Number	Number of Cycles	Temperature	Time
1	1	68°C	2 minutes
2	1	98°C	30 seconds
3	5	98°C	30 seconds
		56°C	30 seconds
		72°C	1 minute
4	1	4°C	Hold

Step 4. Purify amplified DNA using AMPure XP beads

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

Prepare the workstation and reagents

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo decks, and BenchCel with a DNA Away decontamination wipe.
3. Verify that the AMPure XP bead suspension is at room temperature. (If necessary, allow the bead solution to come to room temperature for at least 30 minutes.) *Do not freeze the beads at any time.*
4. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
5. Turn on the ThermoCube, set to 4°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
6. Pre-set the temperature of Bravo deck position 4 to 45°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
7. Prepare a Nunc DeepWell source plate for the beads by adding 45 µL of homogeneous AMPure XP beads per well, for each well to be processed.
8. Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water.
9. Prepare a separate Thermo Scientific reservoir containing 45 mL of freshly-prepared 70% ethanol.
10. Centrifuge the amplified DNA sample plate for 30 seconds to drive the well contents off the walls and plate seal and to remove air bubbles.

11 Load the Labware MiniHub according to Table 23, using the plate orientations shown in Figure 2 on page 20.

Table 23 Initial MiniHub configuration for AMPureXP_QXT_ILM_v1.0.pro:Whole Genome PCR

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

12 Load the Bravo deck according to Table 24.

Table 24 Initial Bravo deck configuration for AMPureXP_QXT_ILM_v1.0.pro:Whole Genome PCR

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

13 Load the BenchCel Microplate Handling Workstation according to Table 25.

Table 25 Initial BenchCel configuration for AMPureXP_QXT_ILM_v1.0.pro:Whole Genome PCR

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

Run VWorks protocol *AMPureXP_QXT_ILM_v1.0.pro:Whole Genome PCR*

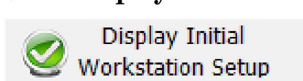
14 On the SureSelect setup form, under **Select Protocol to Run**, select **AMPureXP_QXT_ILM_v1.0.pro:Whole Genome PCR**.

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

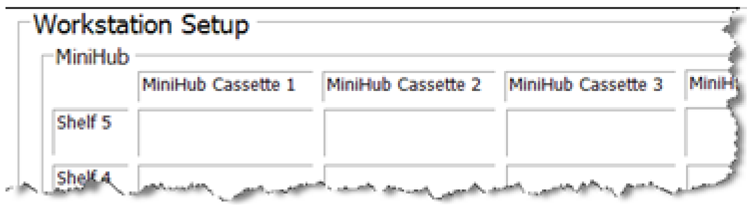
15 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate containing the amplified libraries at position 9.

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

17 Click **Display Initial Workstation Setup**.



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



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

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

Step 5. Assess library DNA quantity and quality using the 2100 Bioanalyzer and High Sensitivity DNA Assay

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

NOTE

Do not use Agilent's DNA 1000 Assay to analyze the whole genome samples. The expected distribution of whole genome library fragment sizes is not compatible with the DNA 1000 Assay. See Figure 6, below, for a sample electropherogram.

The presence of magnetic beads in the samples may adversely impact the Bioanalyzer results. If you suspect bead contamination in the samples, place the plate or strip tube on the magnetic rack before withdrawing samples for analysis.

---

1 Set up the 2100 Bioanalyzer as instructed in the reagent kit guide.
2 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µl of a 1:10 dilution of each sample for the analysis.
3 Load the prepared chip into the instrument and start the run within five minutes after preparation.
4 Analyze the results, using the guidelines below:
    - Typical whole genome library electropherograms show a broad distribution of DNA fragments. A sample electropherogram is shown in Figure 6.
    - Check the Average Size [bp] of DNA fragments reported in the Bioanalyzer results. Sequencing data may be acquired from libraries with a broad range of average fragment sizes. The protocol has been optimized, however, to produce whole genome libraries with average DNA fragment sizes between approximately 600 and 1000 bp.

NOTE

An average fragment size significantly less than 600 bp may indicate too little gDNA in the fragmentation reaction and may be associated with increased duplicates in the sequencing data. In contrast, libraries with an unusually large average fragment size may indicate too much gDNA in the fragmentation reaction and may require higher DNA concentrations for optimal cluster density in the sequencing reaction.

---

5 Measure the concentration of each library by integrating under the entire peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

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

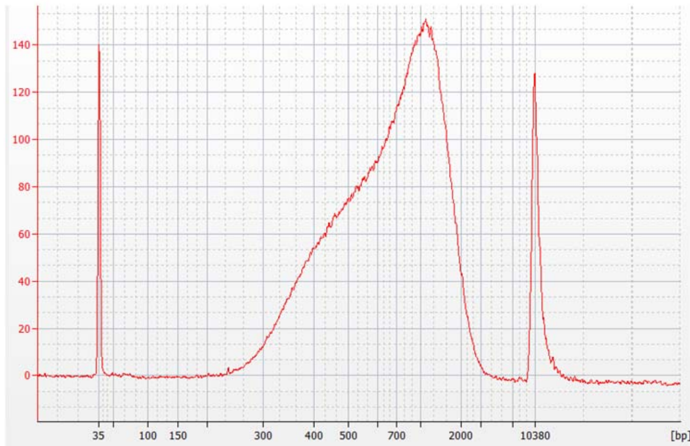


Figure 6 Analysis of amplified library DNA using a High-Sensitivity DNA Assay.

Step 6. Pool samples for multiplexed sequencing

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

Guidelines for optimal low-level multiplexing of samples indexed using the SureSelect^{QXT} dual indexes are provided on page 73.

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

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

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

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

is the number of indexes, and

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

Table 26 shows an example of the amount of 4 index-tagged samples (of different concentrations) and Low TE needed for a final volume of 20 µL at 10 nM.

Table 26 Example of indexed sample volume calculation for total volume of 20 µL

Component	V(f)	C(i)	C(f)	#	Volume to use (µL)
Sample 1	20 µL	20 nM	10 nM	4	2.5
Sample 2	20 µL	10 nM	10 nM	4	5
Sample 3	20 µL	17 nM	10 nM	4	2.9
Sample 4	20 µL	25 nM	10 nM	4	2
Low TE					7.6

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

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

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

Step 7. Prepare sequencing samples

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See Table 27 for kit configurations compatible with the recommended read length plus reads for the SureSelect^{QXT} 8-bp dual indexes.

The optimal seeding concentration for SureSelect^{QXT} whole genome libraries varies according to sequencing platform, run type and Illumina kit version. See Table 27 for guidelines. Seeding concentration and cluster density may also need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality.

To do this step, refer to the manufacturer's instructions, using the modifications described on page 62 for use of the SureSelect^{QXT} Read Primers with the Illumina Paired-End Cluster Generation Kits. Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Table 27 Illumina Kit Configuration Selection Guidelines

Platform	Run Type	Read Length*	SBS Kit Configuration	Chemistry	Seeding Concentration
HiSeq 2500	Rapid Run	2 × 100 bp	200 Cycle Kit	v2	14–20 pM
HiSeq 2500	High Output	2 × 100 bp	250 Cycle Kit	v4	14–20 pM
MiSeq	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	14–20 pM
MiSeq	All Runs	2 × 76 bp	150 Cycle Kit	v3	14–20 pM
NextSeq 500/550	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2.5	2 pM
HiSeq 3000/4000	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1	200–300 pM
NovaSeq 6000	Standard Workflow Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1.0 or v1.5	300–600 pM
NovaSeq 6000	Xp Workflow Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v1.0 or v1.5	200–400 pM

* If your application requires a different read length, verify that you have sufficient sequencing reagents to complete Reads 1 and 2 in addition to the dual 8-bp index reads.

Using the SureSelect^{QXT} Read Primers with Illumina's Paired-End Cluster Generation Kits

To sequence the SureSelect^{QXT} libraries on Illumina's sequencing platforms, you need to use the following custom sequencing primers, provided in SureSelect QXT Library Prep Kit Box 2:

- **SureSelect QXT Read Primer 1**
- **SureSelect QXT Read Primer 2**
- **SureSelect QXT Index 1 Read Primer**
- **SureSelect QXT Index 2 Read Primer** (this primer is used only for HiSeq 3000, HiSeq 4000, and NextSeq platforms and for NovaSeq platform runs using v1.5 chemistry)

These SureSelect^{QXT} custom sequencing primers are provided at 100 µM and must be diluted in the corresponding Illumina primer solution, using the platform-specific instructions below:

For the HiSeq 2500 platform, combine the primers as shown in Table 28 or Table 29 on page 63.

For the HiSeq 3000 or HiSeq 4000 platform, combine the primers as shown in Table 30 on page 63.

For the MiSeq platform, combine the primers as shown in Table 31 on page 64.

For the NextSeq platform, combine the primers as shown in Table 32 or Table 33 on page 64.

For the NovaSeq platform, combine the primers as shown in Table 34 through Table 37 on page 65.

NOTE

It is important to combine the primers precisely in the indicated ratios. Carefully follow the instructions indicated in Table 28 to Table 37. Where specified, add the custom primer volume directly to the solution already in cBot reagent plate wells. Otherwise, combine measured volumes of each solution; do not rely on volumes reported on vial labels or in Illumina literature. Vortex each mixture vigorously to ensure homogeneity for proper detection of the indexes using the custom read primers.

Table 28 HiSeq 2500 High Output custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	Total Volume
Read 1	6 µl SureSelect QXT Read Primer 1 (brown cap)	1194 µl HP10	1.2 ml*
Index	15 µl SureSelect QXT Index 1 Read Primer (clear cap)	2985 µl HP12	3 ml
Read 2	15 µl SureSelect QXT Read Primer 2 (black cap)	2985 µl HP11	3 ml

* Aliquot the mixture as directed for HP6 or HP10 in Illumina's cluster generation protocol.

Table 29 HiSeq 2500 Rapid Mode custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	Total Volume
Read 1	8.8 µl SureSelect QXT Read Primer 1 (brown cap)	1741.2 µl HP10	1.75 ml
Index	8.8 µl SureSelect QXT Index 1 Read Primer (clear cap)	1741.2 µl HP12	1.75 ml
Read 2	8.8 µl SureSelect QXT Read Primer 2 (black cap)	1741.2 µl HP11	1.75 ml

Table 30 HiSeq 3000 and HiSeq 4000 custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	Total Volume	Reagent Rack Position
Read 1	1.5 µl SureSelect QXT Read Primer 1 (brown cap)	298.5 µl HP10*	0.3 ml per well	cBot Column 11
Read 2	15 µl SureSelect QXT Read Primer 2 (black cap)	2985 µl HP11	3 ml	16
Index 1+ Index 2	22.5 µl SureSelect QXT Index 1 Read Primer (clear cap) + 22.5 µl SureSelect QXT Index 2 Read Primer (purple cap)	4455 µl HP14	4.5 ml	17

* Use cBot recipe *HiSeq_3000_4000_HD_Exclusion_Amp_v1.0*. Add 1.5 µl SureSelect QXT Read Primer 1 to the 298.5 µl of HP10 in each well of column 11 in the cBot reagent plate.

Table 31 MiSeq platform custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina TruSeq Primer	Total Volume	Final Cartridge Position
Read 1	3 µl SureSelect QXT Read Primer 1 (brown cap)	597 µl HP10 (well 12)	0.6 ml	well 18
Index	3 µl SureSelect QXT Index 1 Read Primer (clear cap)	597 µl HP12 (well 13)	0.6 ml	well 19
Read 2	3 µl SureSelect QXT Read Primer 2 (black cap)	597 µl HP11 (well 14)	0.6 ml	well 20

Table 32 NextSeq 500/550 High-Output v2 Kit custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	3.9 µl SureSelect QXT Read Primer 1 (brown cap)	1296.1 µl BP10 (from well 20)	1.3 ml	well 7
Read 2	4.2 µl SureSelect QXT Read Primer 2 (black cap)	1395.8 µl BP11 (from well 21)	1.4 ml	well 8
Index 1+ Index 2	6 µl SureSelect QXT Index 1 Read Primer (clear cap) + 6 µl SureSelect QXT Index 2 Read Primer (purple cap)	1988 µl BP14 (from well 22)	2 ml	well 9

Table 33 NextSeq 500/550 Mid-Output v2 Kit custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	2.7 µl SureSelect QXT Read Primer 1 (brown cap)	897.3 µl BP10 (from well 20)	0.9 ml	well 7
Read 2	3.3 µl SureSelect QXT Read Primer 2 (black cap)	1096.7 µl BP11 (from well 21)	1.1 ml	well 8
Index 1+ Index 2	4.8 µl SureSelect QXT Index 1 Read Primer (clear cap) + 4.8 µl SureSelect QXT Index 2 Read Primer (purple cap)	1590.4 µl BP14 (from well 22)	1.6 ml	well 9

Table 34 NovaSeq 6000 using SP/S1/S2 flowcell with v1.0 chemistry custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	12 µl SureSelect QXT Read Primer 1 (brown cap)	3988 µl BP10 (well 24)	4 ml	5
Index	15 µl SureSelect QXT Index 1 Read Primer (clear cap)	4985 µl BP14 (well 23)	5 ml	7
Read 2	6 µl SureSelect QXT Read Primer 2 (black cap)	1994 µl BP11 (well 13)	2 ml	6

Table 35 NovaSeq 6000 using S4 flowcell with v1.0 chemistry custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	21.9 µl SureSelect QXT Read Primer 1 (brown cap)	7278.1 µl BP10 (well 24)	7.3 ml	5
Index	15 µl SureSelect QXT Index 1 Read Primer (clear cap)	4985 µl BP14 (well 23)	5 ml	7
Read 2	10.5 µl SureSelect QXT Read Primer 2 (black cap)	3489.5 µl BP11 (well 13)	3.5 ml	6

Table 36 NovaSeq 6000 using SP/S1/S2 flowcell with v1.5 chemistry custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	12 µl SureSelect QXT Read Primer 1 (brown cap)	3988 µl BP10 (well 24)	4 ml	5
Index 1+ Index 2	15 µl SureSelect QXT Index 1 Read Primer (clear cap) + 15 µl SureSelect QXT Index 2 Read Primer (purple cap)	4970 µl VP14 (well 23)	5 ml	7
Read 2	6 µl SureSelect QXT Read Primer 2 (black cap)	1994 µl BP11 (well 13)	2 ml	6

Table 37 NovaSeq 6000 using S4 flowcell with v1.5 chemistry custom sequencing primer preparation

Sequencing Read	Volume of SureSelect^{QXT} Primer	Volume of Illumina Primer	Total Volume	Final Cartridge Position
Read 1	21.9 µl SureSelect QXT Read Primer 1 (brown cap)	7278.1 µl BP10 (well 24)	7.3 ml	5
Index 1+ Index 2	15 µl SureSelect QXT Index 1 Read Primer (clear cap) + 15 µl SureSelect QXT Index 2 Read Primer (purple cap)	4970 µl VP14 (well 23)	5 ml	7
Read 2	10.5 µl SureSelect QXT Read Primer 2 (black cap)	3489.5 µl BP11 (well 13)	3.5 ml	6

Step 8. Set up the sequencing run and trim adaptors from the reads

Refer to Illumina protocols to set up custom sequencing primer runs. Before aligning reads to the reference genome, SureSelect^{QXT} adaptor sequences must be trimmed from the reads using the additional platform-specific guidelines below.

For SureSelect^{QXT} dual index sequence information, see page 71.

MiSeq platform sequencing run setup and adaptor trimming guidelines

Use the Illumina Experiment Manager (IEM) software to generate a custom primer Sample Sheet.

Set up the run to include adapter trimming using the IEM Sample Sheet Wizard. When prompted by the wizard, select the *Use Adapter Trimming* option, and specify **CTGTCTCTTGATCACA** as the adapter sequence. This enables the MiSeq Reporter software to identify the adaptor sequence and trim the adaptor from reads.

HiSeq/NextSeq/NovaSeq platform sequencing run setup and adaptor trimming guidelines

Set up sequencing runs using the settings shown in Table 38. For HiSeq runs, select *Dual Index* on the *Run Configuration* screen of the instrument control software interface. Since custom primers are spiked into the standard sequencing primer tubes, no additional specialized settings are required to accommodate the use of custom primers in the run.

For the NextSeq or NovaSeq platform, Cycle Number and custom sequencing primer settings can be specified on the *Run Configuration* screen of the instrument control software interface.

Table 38 Run Configuration screen Cycle Number settings

Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	8
Index 2 (i5)	8
Read 2	100

After the sequencing run is complete, generate demultiplexed FASTQ data following Illumina's instructions and then trim adaptor sequences from the reads using the Trimmer utility of the Agilent Genomics NextGen Toolkit (AGeNT). For additional information and to download this toolkit free-of-charge, visit the AGeNT page at www.agilent.com.

4
Reference

This chapter contains reference information, including component kit contents and reference information for use during the downstream sample sequencing steps.

Kit Contents

SureSelect^{QXT} Library Prep Kits contain the following component kits:

Table 39 SureSelect^{QXT} Library Prep Kit Contents

Component Kits	Storage Condition	Component Kit p/n
SureSelect QXT Library Prep Kit, Box 1	Room Temperature	5500-0119
SureSelect QXT Library Prep Kit Box 2	–20°C	5500-0127

The contents of each of the component kits listed in Table 39 are described in Table 40 and Table 41 below.

Table 40 SureSelect QXT Library Prep Kit, Box 1 Content

Kit Component	Format
SureSelect QXT Stop Solution	bottle

Table 41 SureSelect QXT Library Prep Kit Box 2 Content

Kit Component	Format
SureSelect QXT Buffer	bottle
SureSelect QXT Enzyme Mix ILM	tube with orange cap
Herculase II Fusion DNA Polymerase	tube with red cap
Herculase II 5× Reaction Buffer	tube with clear cap
100 mM dNTP Mix (25 mM each dNTP)	tube with green cap
DMSO	tube with green cap
SureSelect QXT Read Primer 1	tube with amber cap
SureSelect QXT Read Primer 2	tube with black cap
SureSelect QXT Index 1 Read Primer	tube with clear cap
SureSelect QXT Index 2 Read Primer	tube with purple cap
SureSelect QXT P7 dual indexing primers	P7 i1 through P7 i12 provided in 12 tubes with yellow caps (one tube per primer)
SureSelect QXT P5 dual indexing primers	P5 i13 through P5 i20 provided in 8 tubes with blue caps (one tube per primer)

Nucleotide Sequences of SureSelect^{QXT} Dual Indexes

The nucleotide sequence of each SureSelect^{QXT} index is provided in the tables below.

Note that some index number assignments of the SureSelect^{QXT} P5 and P7 indexes differ from the index numbers assignments used by Illumina for indexes of similar or identical sequence.

Each index is 8 bases in length. Refer to Illumina's sequencing run setup instructions for sequencing libraries using 8-base indexes.

Table 42 SureSelect^{QXT} P7 Indexes 1 to 12

Index Name with Number	Sequence
P7 Index 1 (P7 i1)	TAAGGCGA
P7 Index 2 (P7 i2)	CGTACTAG
P7 Index 3 (P7 i3)	AGGCAGAA
P7 Index 4 (P7 i4)	TCCTGAGC
P7 Index 5 (P7 i5)	GTAGAGGA
P7 Index 6 (P7 i6)	TAGGCATG
P7 Index 7 (P7 i7)	CTCTCTAC
P7 Index 8 (P7 i8)	CAGAGAGG
P7 Index 9 (P7 i9)	GCTACGCT
P7 Index 10 (P7 i10)	CGAGGCTG
P7 Index 11 (P7 i11)	AAGAGGCA
P7 Index 12 (P7 i12)	GGACTCCT

Table 43 SureSelect^{QXT} P5 Indexes 13 to 20 for HiSeq 2500, MiSeq, or NovaSeq (v1.0 chemistry) platform

Index Number	Sequence
P5 Index 13 (P5 i13)	TAGATCGC
P5 Index 14 (P5 i14)	CTCTCTAT
P5 Index 15 (P5 i15)	TATCCTCT
P5 Index 16 (P5 i16)	AGAGTAGA
P5 Index 17 (P5 i17)	GTAAGGAG
P5 Index 18 (P5 i18)	ACTGCATA
P5 Index 19 (P5 i19)	AAGGAGTA
P5 Index 20 (P5 i20)	CTAAGCCT

Table 44 SureSelect^{QXT} P5 Indexes 13 to 20 for HiSeq 3000/4000, NextSeq, or NovaSeq (v1.5 chemistry) platform*

Index Number	Sequence
P5 Index 13 (P5 i13)	GCGATCTA
P5 Index 14 (P5 i14)	ATAGAGAG
P5 Index 15 (P5 i15)	AGAGGATA
P5 Index 16 (P5 i16)	TCTACTCT
P5 Index 17 (P5 i17)	CTCCTTAC
P5 Index 18 (P5 i18)	TATGCAGT
P5 Index 19 (P5 i19)	TACTCCTT
P5 Index 20 (P5 i20)	AGGCTTAG

* When doing runs on these platforms through BaseSpace, use the reverse complement sequences provided in Table 43.

Guidelines for Multiplexing with Dual-Indexed Samples

Agilent recommends following the dual index sample pooling guidelines shown in Table 45. These are designed to maintain color balance at each cycle of the index reads on both ends. They also provide flexibility of demultiplexing as single or dual indexed samples in low-plexity experiments. One-base mismatches should be allowed during demultiplexing.

Table 45 Dual index sample pooling guidelines for 96 Reaction Kits

Plexity of Sample Pool	Recommended SureSelectQXT P7 Indexes	Recommended SureSelectQXT P5 Indexes
1-plex	Any P7 index i1 to i12	Any P5 index (i13 to i20)
2-plex	P7 i1 and P7 i2 OR P7 i2 and P7 i4	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18
3-plex	P7 i1, P7 i2 and P7 i4 OR P7 i3, P7 i4 and P7 i6 OR P7 i5, P7 i7 and P7 i8	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
4-plex	P7 i1, P7 i2, P7 i3* and P7 i4 OR P7 i3, P7 i4, P7 i5* and P7 i6 OR P7 i5, P7 i6*, P7 i7 and P7 i8	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
5-plex	P7 i1, P7 i2, P7 i3*, P7 i4 and P7 i5* OR P7 i3, P7 i4, P7 i5*, P7 i6 and p7 i7* OR P7 i5, P7 i6*, P7 i7, P7 i8 and p7 i9*	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
6- to 12-plex	Any combination of P7 indexes i1 to i12 using each index only once	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
13-to 96-plex	All twelve P7 indexes (i1 to i12)	P5 i13 and P5 i14 and any other P5 index OR P5 i15 and P5 i16 and any other P5 index OR P5 i17 and P5 i18 and any other P5 index (as needed)

* The indicated indexes may be substituted with another index, as long as the substitute index differs from all others used in the sample pool.

In This Book

This guide contains information to run the SureSelect^{QXT} Automated Library Prep protocol.

Version E0, January 2021

p/n G9682-90010

Agilent Technologies