



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

**Featuring Transposase-Based Library
Prep Technology**

**Automated using Agilent NGS Bravo
Option A**

Protocol

Version B0, November 2015

**SureSelect platform manufactured with Agilent
SurePrint Technology**

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



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In this Guide...

This guide describes an optimized protocol for Illumina paired-end multiplexed library preparation using the SureSelect^{QXT} Automated Whole Genome Library Prep system.

Sample processing steps are automated using Agilent's NGS Bravo Option A.

1 Before You Begin

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

2 Using the Agilent NGS Bravo Option A for SureSelect^{QXT} Whole Genome Library Prep

This chapter contains an orientation to the Agilent NGS Bravo, an overview of the SureSelect whole genome library prep protocol, and considerations for designing SureSelect experiments for automated processing using the Agilent NGS Bravo Option A.

3 Sample Preparation

This chapter describes the steps to prepare gDNA sequencing libraries for whole genome library prep.

4 Reference

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

What's New in Version B0

- Updates to custom sequencing primer mixtures for the NextSeq 500 v2 platform (see [Table 29](#) and [Table 30](#) on page 63)
- Update to P5 Index details for NextSeq 500 platform runs using BaseSpace (see [Table 37](#) on page 71)
- Update to Qubit dsDNA Assay Kit nomenclature ([Table 1](#) on page 12 and [step 4](#) on [page 34](#))
- Updates to SureCycler 8800 PCR plate compatibility considerations (see *Caution* on [page 31](#))
- Correction to ordering information for Axygen 96 Deep Well plates (see [Table 2](#) on page 13)
- Revised ordering information for nucleic acid surface decontamination wipes ([Table 2](#) on page 13)

What's New in Version A1

- Support for sequencing using NextSeq 500 v2 (see [page 60](#) to [page 62](#)) and updates to general sequencing guidelines (see [page 64](#) to [page 65](#)).
- Updates to sequencing run setup instructions for 8 bp dual indexes (see [Table 31](#) on page 66).
- Update to Qubit dsDNA Assay Kit ordering information ([Table 1](#) on page 12).

Content

1	Before You Begin	9
	Procedural Notes	10
	Safety Notes	11
	Required Reagents	12
	Required Equipment	13
2	Using the Agilent NGS Bravo Option A for SureSelect^{QXT} Whole Genome Library Prep	15
	About the Agilent NGS Bravo Option A	16
	About the Bravo Platform	16
	VWorks Automation Control Software	20
	Overview of the SureSelect ^{QXT} Whole Genome Library Prep Procedure	27
	Experimental Setup Considerations for Automated Runs	29
	Considerations for Placement of gDNA Samples in 96-well Plates for Automated Processing	30
	Considerations for Equipment Setup	30
	PCR Plate Type Considerations	31
3	Sample Preparation	33
	Step 1. Prepare the genomic DNA samples and Library Prep reagents	34
	Step 2. Fragment and adaptor-tag the genomic DNA samples	36
	Step 3. Purify adaptor-tagged DNA using AMPure XP beads	41
	Step 4. Amplify adaptor-ligated libraries	44
	Step 5. Purify amplified DNA using AMPure XP beads	53
	Step 6. Assess library DNA quantity and quality using the 2100 Bioanalyzer and High Sensitivity DNA Assay	56
	Step 7. Pool samples for multiplexed sequencing	58
	Step 8. Prepare sequencing samples	60
	Step 9. Set up the sequencing run and trim adaptors from the reads	64

Contents

4	Reference	67
	Kit Contents	68
	Nucleotide Sequences of SureSelect ^{0XT} Dual Indexes	70
	Guidelines for Multiplexing with Dual-Indexed Samples	72



1 Before You Begin

Procedural Notes	10
Safety Notes	11
Required Reagents	12
Required Equipment	13

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

NOTE

This protocol describes automated sample processing using the Agilent NGS Bravo Option A. For automated sample processing using the Agilent NGS Workstation Option B, see publication G9682-90010. For non-automated sample processing procedures see publication G9682-90000.

NOTE

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



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.
- Certain protocol steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the Agilent NGS Bravo to allow rapid and efficient plate transfer.
- 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 (BL1) safety rules.

Safety Notes

CAUTION

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

1 Before You Begin
Required Reagents

Required Reagents

Table 1 Required Reagents for SureSelect^{0XT} Whole Genome Library Prep

Description	Vendor and part number
SureSelect ^{0XT} Library Prep Kit	Agilent
Illumina HiSeq or MiSeq platform (ILM), 96 Samples	p/n G9682B
Illumina NextSeq platform (NSQ), 96 Samples	p/n G9684B
Agencourt AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881
450 mL	p/n A63882
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Life Technologies p/n 12090-015, or equivalent
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Qubit dsDNA HS Assay Kit <i>or</i>	Life Technologies p/n Q32851
Qubit dsDNA BR Assay Kit	Life Technologies
100 assays	p/n Q32850
500 assays	p/n Q32853
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930

Required Equipment

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

Description	Vendor and part number
Agilent NGS Bravo Option A 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
PlateLoc Thermal Microplate Sealer with Small Hotplate	Agilent p/n G5402#226
Clear Peelable Seal plate seals (for use with the PlateLoc Thermal Plate Sealer)	Agilent p/n 16985-001
Thermal cycler and accessories	SureCycler 8800 Thermal Cycler (Agilent p/n G8810A), 96 well plate module (Agilent p/n G8810A) and compression mats (Agilent p/n 410187) or equivalent
PCR plates compatible with selected Thermal Cycler, e.g. Agilent semi-skirted PCR plate for the SureCycler 8800 Thermal Cycler When selecting plates for another thermal cycler, see Table 6 on page 31 for the list of PCR plates supported in automation protocols	Agilent p/n 401334
Eppendorf twin.tec full-skirted 96-well PCR plates	Eppendorf p/n 951020401 or 951020619
Thermo Scientific Reservoirs	Thermo Scientific p/n 1064156
Nunc DeepWell Plates, sterile, 1.3-mL well volume	Thermo Scientific p/n 260251
Axygen 96 Deep Well Plate, 2 mL, Square Well (waste reservoirs; working volume 2.2 mL)	Axygen p/n P-2ML-SQ-C E & K Scientific p/n EK-2440
Nucleic acid surface decontamination wipes	DNA Away Surface Decontaminant Wipes, Thermo Scientific p/n 7008, or equivalent
Qubit Fluorometer	Life Technologies p/n Q32857
Qubit Assay Tubes	Life Technologies p/n Q32856

1 Before You Begin

Required Equipment

Table 2 Required Equipment for SureSelect^{0XT} Whole Genome Library Prep

Description	Vendor and part number
DNA Analysis Platform and Consumables	
Agilent 2100 Bioanalyzer Laptop Bundle	Agilent p/n G2943CA
Agilent 2100 Bioanalyzer Electrophoresis Set	Agilent p/n G2947CA
Agilent DNA 1000 Kit	Agilent p/n 5067-1504
Agilent High Sensitivity DNA Kit	Agilent p/n 5067-4626
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	
	Pipetman or equivalent
P10, P20, P200 and P1000 pipettes	
	Pipetman P10, P20, P200, P1000 or equivalent
Vortex mixer	
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	



2 Using the Agilent NGS Bravo Option A for SureSelect^{QXT} Whole Genome Library Prep

About the Agilent NGS Bravo Option A	16
Overview of the SureSelect ^{QXT} Whole Genome Library Prep Procedure	27
Experimental Setup Considerations for Automated Runs	29

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



About the Agilent NGS Bravo Option A

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 μ L to 250 μ L.

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.

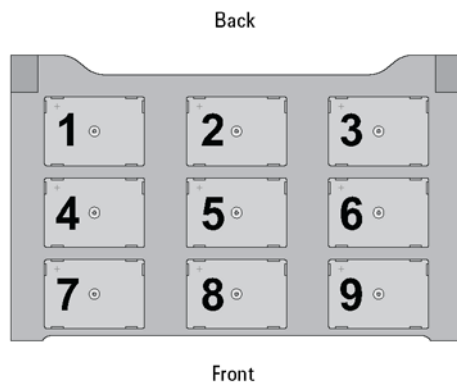


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

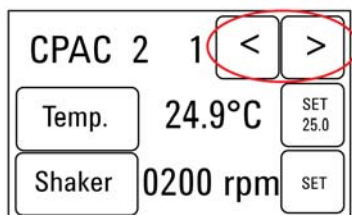
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the 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 3](#) for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

Table 3 Inheco Multi TEC Control touchscreen designations

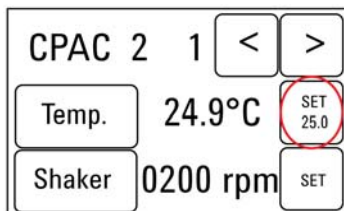
Bravo Deck Position	Designation on Inheco Multi TEC Control Screen
4	CPAC 2 1
6	CPAC 2 2

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

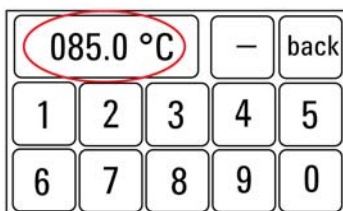


2 Using the Agilent NGS Bravo Option A for SureSelect^{0XT} Whole Genome Library Prep About the Bravo Platform

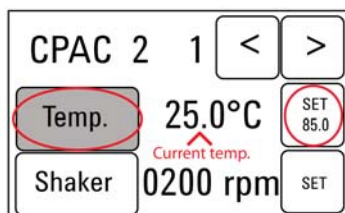
- 2 To set the temperature of the selected block, press the SET button.



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



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



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

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

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

- 1 Turn on the ThermoCube and wait for the LCD screen to display **TEMP.**
- 2 Press the **UP** or **DOWN** button to change **SET TEMP 1** to the required set point.
- 3 Press the **START** button.

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

VWorks Automation Control Software

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

NOTE

The instructions in this manual are compatible with VWorks software version 11.3.0.1195, including SureSelect^{0XT} 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 QXT_ILM_OptA_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.)

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

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

Current Tip State

Select columns of unused tips (Box 2)

Select columns of used tips (Box 8)

Reset **Clear**

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

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

NOTE

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

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

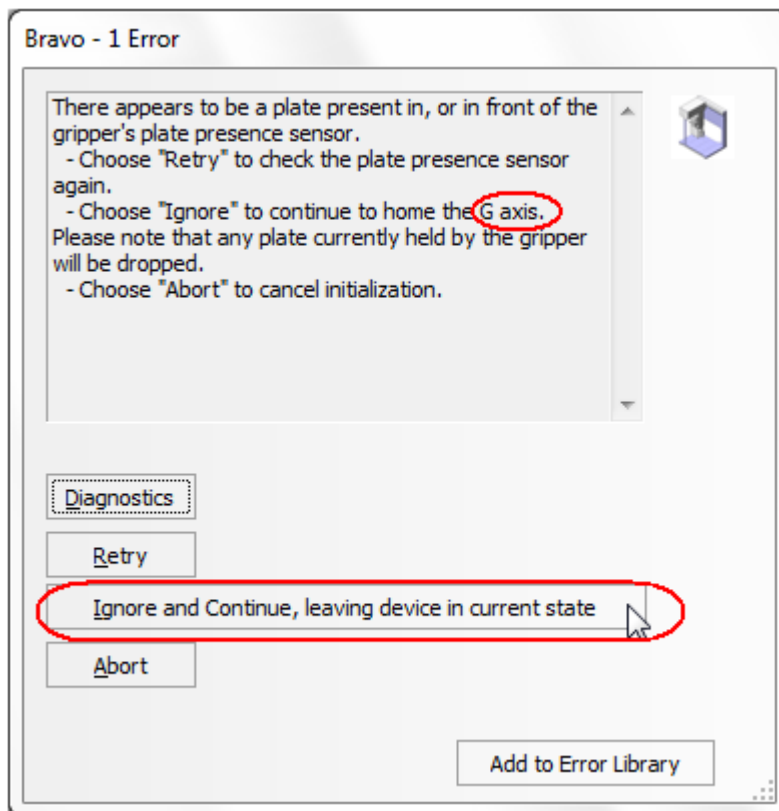
- 6 After verifying that the NGS Bravo has been set up correctly, click **Run Selected Protocol**.



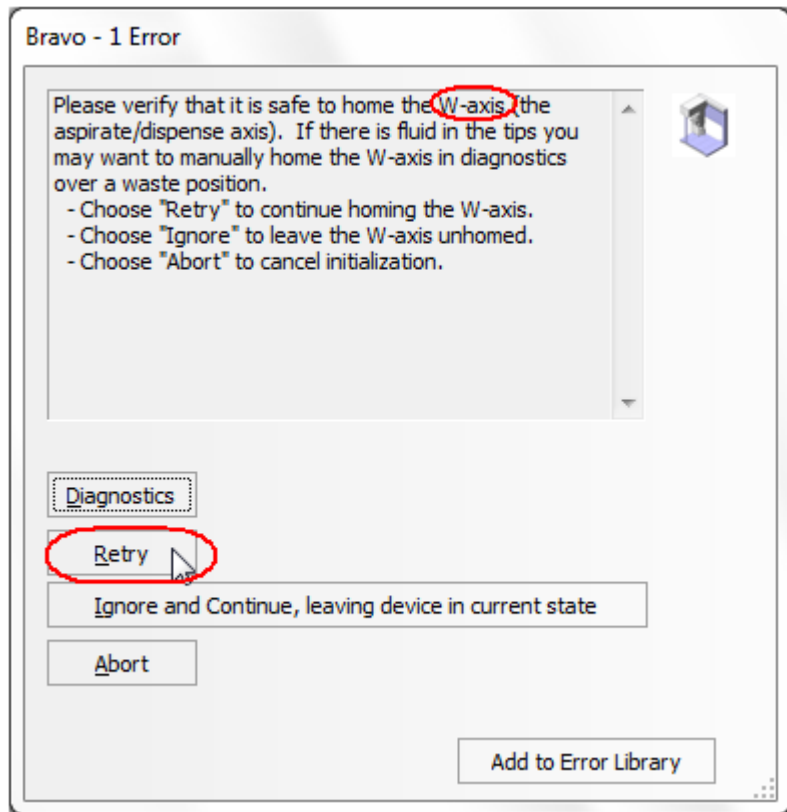
Error messages encountered at start of run

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

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



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



Verifying the Simulation setting

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

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



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

NOTE

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

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

Figure 2 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 4 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.

2 Using the Agilent NGS Bravo Option A for SureSelect^{QXT} Whole Genome Library Prep
 Overview of the SureSelect^{QXT} Whole Genome Library Prep Procedure

SureSelect^{QXT}
 Whole Genome
 NGS Workflow

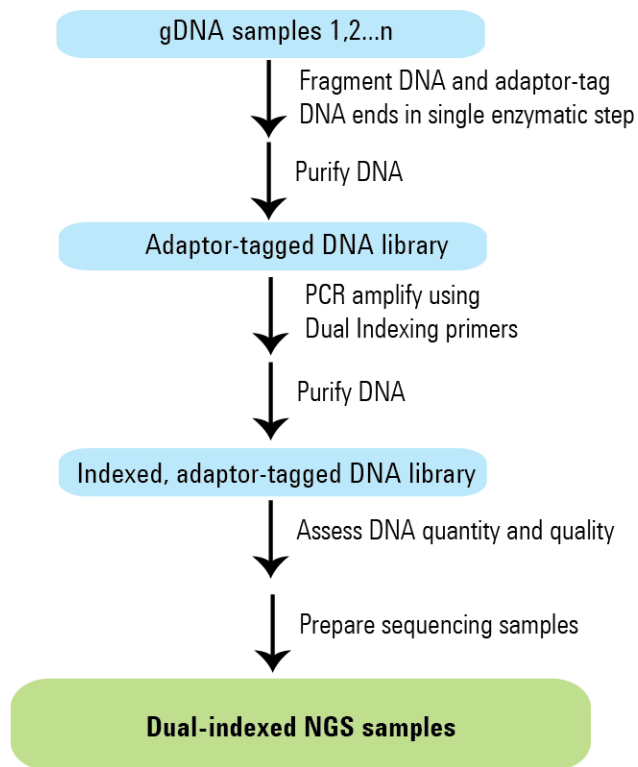


Figure 2 Overall sequencing sample preparation workflow.

Table 4 Overview of VWorks protocols used for SureSelect^{QXT} Whole Genome Library Prep using NGS Bravo Option A

Workflow Step	VWorks Protocols Used for Agilent NGS Bravo Option A automation
Prepare fragmented and adaptor-tagged DNA	01 Tn_QXT_ILM_v1.0.pro
Purify DNA using AMPure XP beads	02 Cleanup_Tn_v1.0.pro
Amplify adaptor-tagged DNA	03a WholeGenomePCR_QXT_ILM_v1.0.pro
Purify DNA using AMPure XP beads	04a Cleanup_WholeGenomePCR_QXT_ILM_v1.0.pro

Experimental Setup Considerations for Automated Runs

Agilent SureSelect automated 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 5 Columns to Samples Equivalency

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

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

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

- The NGS Bravo processes samples column-wise beginning at column 1. gDNA samples should be loaded into 96-well plates column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12. When processing partial runs with <12 sample columns, do not leave empty columns between sample columns; always load the plate using the left-most column that is available.
- 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 2](#)). It is important to review and understand the guidelines for assignment of dual indexing primers on [page 47](#) 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 Bravo to allow rapid and efficient plate transfer.
- Several workflow steps require that the sample plate be sealed using the PlateLoc thermal microplate sealer and then centrifuged to collect any dispersed liquid. To maximize efficiency, locate the centrifuge in close proximity to the Agilent NGS Bravo.

PCR Plate Type Considerations

Automation protocols include several liquid-handling steps in which reagents are dispensed to PCR plates in preparation for transfer to a thermal cycler. For these steps you must specify the PCR plate type to be used on the QXT_ILM_OptA_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 6](#).

2) Select PCR Plate labware for Thermal Cycling

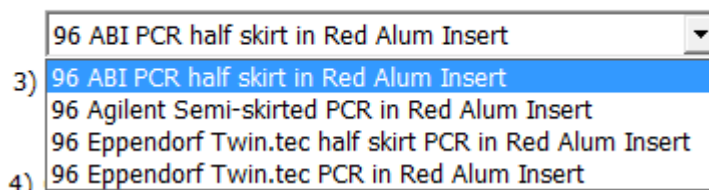


Table 6 Ordering information for supported PCR plates

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

CAUTION

The plates listed in [Table 6](#) are compatible with the Agilent NGS Bravo and associated VWorks automation protocols, designed to support use of various thermal cyclers.

Accordingly, some plates listed in [Table 6](#) are not compatible with the recommended SureCycler 8800 Thermal Cycler. When using the SureCycler 8800 Thermal Cycler in the SureSelect automation workflow, use 96 Agilent semi-skirted PCR plates.

When using a different thermal cycler in the workflow, be sure to select a PCR plate that is compatible with your thermal cycler and that is listed in [Table 6](#).

2 Using the Agilent NGS Bravo Option A for SureSelect^{0XT} Whole Genome Library Prep PCR Plate Type Considerations



3 Sample Preparation

- Step 1. Prepare the genomic DNA samples and Library Prep reagents 34
- Step 2. Fragment and adaptor-tag the genomic DNA samples 36
- Step 3. Purify adaptor-tagged DNA using AMPure XP beads 41
- Step 4. Amplify adaptor-ligated libraries 44
- Step 5. Purify amplified DNA using AMPure XP beads 53
- Step 6. Assess library DNA quantity and quality using the 2100 Bioanalyzer and High Sensitivity DNA Assay 56
- Step 7. Pool samples for multiplexed sequencing 58
- Step 8. Prepare sequencing samples 60
- Step 9. Set up the sequencing run and trim adaptors from the reads 64

This section contains instructions for gDNA library preparation specific to the Illumina paired-read sequencing platform and to automated processing using the Agilent NGS Bravo Option A.



3 Sample Preparation

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

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 46](#).
- 2 Prepare reagents for the purification protocols on [page 41](#) 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 90 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 37](#).

- 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

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

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/ μ L in a 1.5-mL LoBind tube.
 - c** Carefully measure the DNA concentration of each of the 100 ng/ μ L 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/ μ L in a 1.5-mL LoBind tube.
- 5** Transfer 5 μ L of the 10 ng/ μ L-DNA samples into the wells of a 96-well Eppendorf plate, column-wise, for processing on the NGS Bravo, 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.

- 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 39](#).

3 Sample Preparation

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

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

In this step, automation protocol 01 Tn_QXT_ILM_v1.0.pro is used to enzymatically fragment the gDNA and to add adaptors to ends of the fragments in a single reaction. This step uses the SureSelect^{QXT} Reagent Kit components listed in [Table 7](#) in addition to reagents prepared for use on [page 34](#) to [page 35](#).

Table 7 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 NGS Bravo

- 1 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
- 2 Pre-set the temperature of Bravo deck positions 4, 6, and 9 as indicated in [Table 8](#). See [page 17](#) to [page 19](#) for more information on how to do this step.

Table 8 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
4	52°C	Inheco Multi TEC control touchscreen (CPAC 2-1)
6	4°C	Inheco Multi TEC control touchscreen (CPAC 2-2)
9	0°C	ThermoCube control panel

- 3 Place red PCR plate inserts at Bravo deck positions 4 and 9.

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

Prepare the Library Prep Master Mix and Stop Solution source plates

- 4 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 Bravo deck position 3.
- 5 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 6](#) 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.

- 6 Prepare the appropriate volume of Library Prep Master Mix, according to [Table 9](#). Mix well by vortexing for 20 seconds and then keep on ice.

Table 9 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	242.4 μL	403.9 μL	565.4 μL	726.9 μL	1049.9 μL	2099.5 μL

3 Sample Preparation

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

- 7 Prepare the Library Prep master mix source plate using a Nunc DeepWell plate, containing the mixture from [step 6](#). Add the volume indicated in [Table 10](#) 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 3](#).

Table 10 Preparation of the Master Mix Source Plate for 01 Tn_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
Library Prep Master Mix	Column 1 (A1-H1)	27.9 μ L	48.1 μ L	68.3 μ L	88.5 μ L	128.8 μ L	260.1 μ L

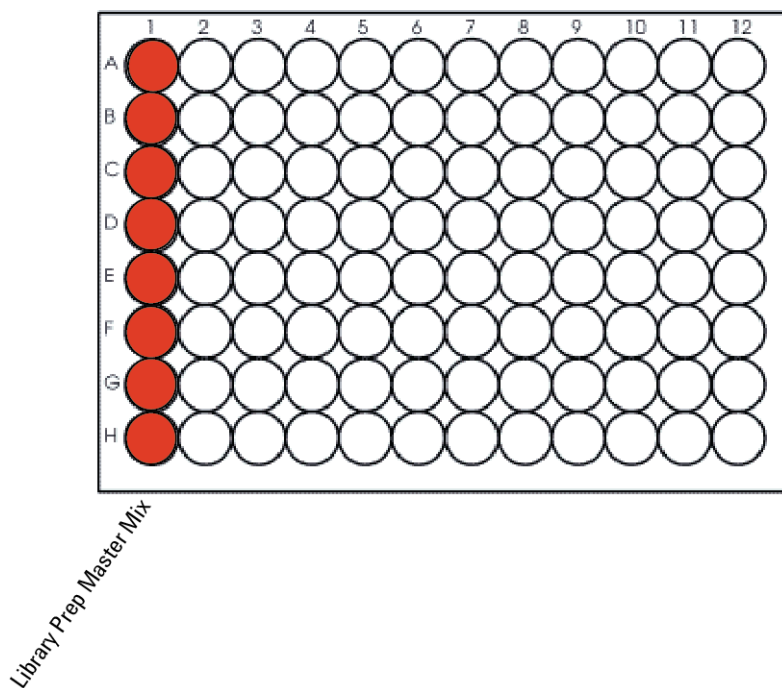


Figure 3 Configuration of the master mix source plate for 01 Tn_QXT_ILM_v1.0.pro

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

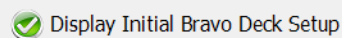
- 8 Seal the master mix source plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
- 9 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles. Keep the master mix source plate on ice.

NOTE

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

Setup and run VWorks protocol 01 Tn_QXT_ILM_v1.0.pro

- 10 On the SureSelect setup form, under **Select Protocol**, select **01 Tn_QXT_ILM_v1.0.pro**.
- 11 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
- 12 Click **Display Initial Bravo Deck Setup**.



- 13 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in [Table 11](#).

Table 11 Initial Bravo deck configuration for 01 Tn_QXT_ILM_v1.0.pro

Location	Content
1	–(empty)–
2	New tip box
3	Stop Solution in Eppendorf plate
4	Empty red insert
5	–(empty)–
6	Library Prep Master Mix source plate (unsealed)
7	gDNA samples (5 µL of 10 ng/µL DNA per well) in Eppendorf plate (unsealed)
8	Empty tip box
9	Empty Eppendorf plate on red insert

3 Sample Preparation

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

14 Verify that the Current Tip State indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See [page 23](#) for more information on using this segment of the form during the run.

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



Running the 01 Tn_QXT_ILM_v1.0.pro protocol takes approximately 20 minutes. Once complete, the adaptor-tagged DNA samples are located in the Eppendorf plate at position 6 of the Bravo deck.

Step 3. Purify adaptor-tagged DNA using AMPure XP beads

This step uses automation protocol **02 Cleanup_Tn_v1.0.pro**.

In this step the NGS Bravo combines the adaptor-tagged samples with AMPure XP beads and then collects and washes the bead-bound DNA. The purified eluted DNA is transferred to an Eppendorf twin.tec plate for further processing.

Prepare the NGS Bravo and reagents

- 1 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
- 2 Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in [Table 12](#). See [page 17](#) for more information on how to do this step.

Table 12 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
4	45°C	Inheco Multi TEC control touchscreen (CPAC 2-1)
6	20°C	Inheco Multi TEC control touchscreen (CPAC 2-2)

- 3 Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.
- 4 Prepare a Nunc DeepWell source plate for the beads by adding 52 μ L of homogeneous AMPure XP beads per well, for each well to be processed.
- 5 Prepare a Thermo Scientific Matrix reservoir containing 15 mL of nuclease-free water.
- 6 Prepare a separate Thermo Scientific Matrix reservoir containing 45 mL of freshly-prepared 70% ethanol.

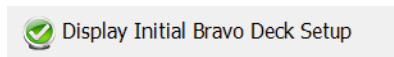
Setup and Run VWorks protocol 02 Cleanup_Tn_v1.0.pro

- 7 On the SureSelect setup form, under **Select Protocol**, select **02 Cleanup_Tn_v1.0.pro**.
- 8 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

3 Sample Preparation

Step 3. Purify adaptor-tagged DNA using AMPure XP beads

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



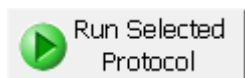
10 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in [Table 13](#).

Table 13 Initial Bravo deck configuration for 02 Cleanup_Tn_v1.0.pro

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
2	New tip box
3	–(empty)–
4	–(empty)–
5	AMPure XP beads in Nunc DeepWell plate (52 µL beads per processing well)
6	Adaptor-tagged DNA samples in Eppendorf twin.tec plate
7	–(empty)–
8	Empty tip box
9	70% ethanol in Matrix reservoir

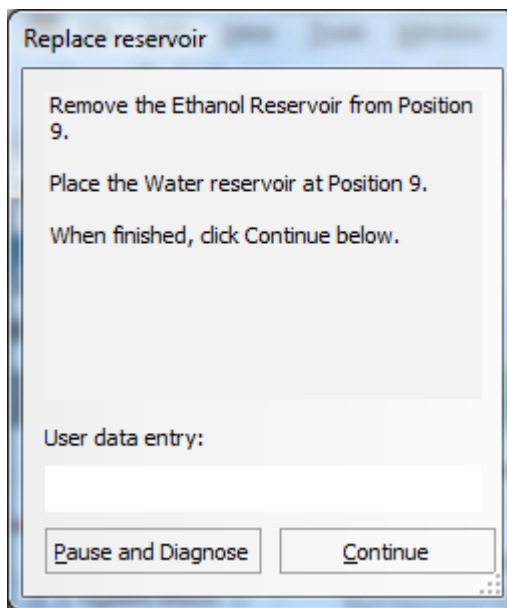
11 Verify that the Current Tip State indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See [page 23](#) for more information on using this segment of the form during the run.

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



Step 3. Purify adaptor-tagged DNA using AMPure XP beads

Running the 02 Cleanup_Tn_v1.0.pro protocol takes approximately 45 minutes. An operator must be present during the run to complete tip box replacement and to transfer the water reservoir to the Bravo deck, when directed by the VWorks prompt shown below.



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

3 Sample Preparation

Step 4. Amplify adaptor-ligated libraries

Step 4. Amplify adaptor-ligated libraries

In this step, the Agilent NGS Bravo completes the liquid handling steps for index tagging and amplification of the adaptor-ligated DNA samples using automation protocol 03a 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 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 47
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 NGS Bravo

- 1 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
- 2 Pre-set the temperature of Bravo deck positions 6 and 9 as indicated in [Table 15](#). See [page 17](#) to [page 19](#) for more information on how to do this step.

Table 15 Bravo Deck Temperature Presets

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

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

Prepare the PCR master mix and master mix source plate

- 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

3 Sample Preparation

Step 4. Amplify adaptor-ligated libraries

- 5 Using the same Nunc DeepWell master mix source plate that was used for the 01 Tn_QXT_ILM_v1.0.pro run, add the volume of PCR Master Mix indicated in [Table 17](#) to all wells of column 2 of the master mix source plate. Keep the source plate on ice until it is used on [page 48](#).

Table 17 Preparation of the Master Mix Source Plate for 03a 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 μ L	54.4 μ L	77.3 μ L	100.1 μ L	145.8 μ L	294.3 μ L

NOTE

If you are using a new DeepWell plate for the PCR Master Mix source plate, leave column 1 empty and add the PCR Master Mix to column 2 of the new plate.

Assign and aliquot dual indexing primers

- 6 Determine the appropriate index assignments for each sample. See the [Reference](#) section for sequences of the index portion of the P7 and P5 indexing primers used to amplify the DNA libraries in this step. (See [Table 36](#) and [Table 37](#) for sequencing on HiSeq and MiSeq platforms or see [Table 36](#) and [Table 38](#) for sequencing on the NextSeq platform.)

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 9](#), 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 7](#) and [step 8](#) 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 39](#) on page 72 for additional details.

- 7 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

3 Sample Preparation

Step 4. Amplify adaptor-ligated libraries

- 8 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.

- 9 Using the Nunc DeepWell master mix source plate containing PCR Master Mix from [step 5](#), 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 4](#). 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.

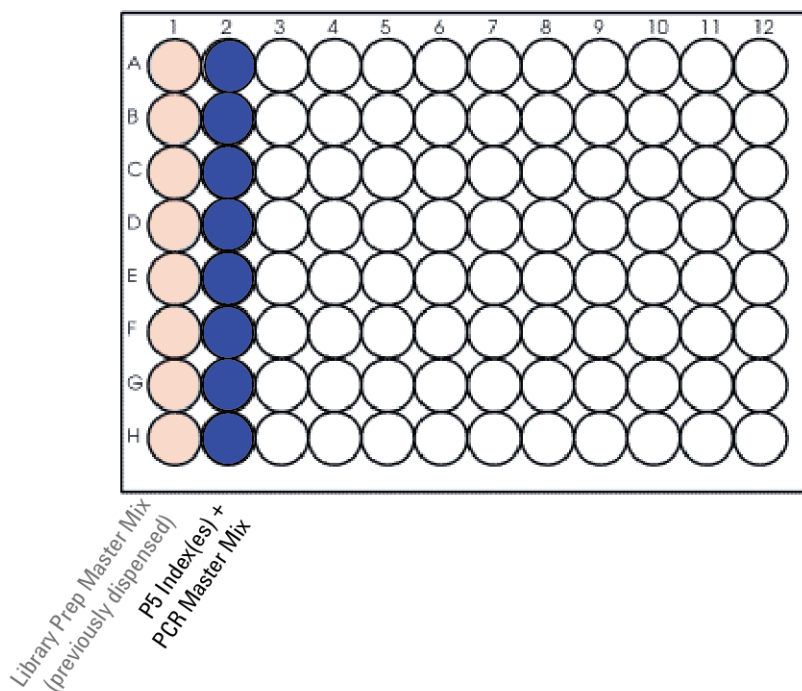


Figure 4 Configuration of the master mix source plate for 03a WholeGenomePCR_QXT_ILM_v1.0.pro. Column 1 was used to dispense master mix during the previous protocol.

- 10** Seal the master mix source plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Vortex the source plate to ensure mixing of the P5 Index and PCR Master Mix solutions.
- 11** Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

NOTE

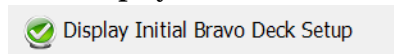
The presence of bubbles in source plate solutions may cause inaccurate volume transfer by the Bravo liquid handling platform. Ensure that the source plate is sealed and centrifuged prior to use in a run.

3 Sample Preparation

Step 4. Amplify adaptor-ligated libraries

Setup and run VWorks protocol 03a WholeGenomePCR_QXT_ILM_v1.0.pro

- 12 On the SureSelect setup form, under **Select Protocol**, select **03a WholeGenomePCR_QXT_ILM_v1.0.pro**.
- 13 Under **Select PCR plate Labware**, select the specific type of PCR plate to be used for thermal cycling (placed at position 6 of the Bravo deck).
- 14 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
- 15 Click **Display Initial Bravo Deck Setup**.



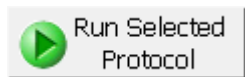
- 16 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in [Table 20](#).

Table 20 Initial Bravo deck configuration for 01 Tn_QXT_ILM_v1.0.pro

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
2	New tip box
3	–(empty)–
4	–(empty)–
5	–(empty)–
6	P7 indexing primers in PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
7	Purified adaptor-tagged DNA samples in Nunc DeepWell plate
8	Empty tip box
9	Master mix plate containing P5 indexing primers and PCR Master Mix in Column 2 (unsealed), seated in silver insert

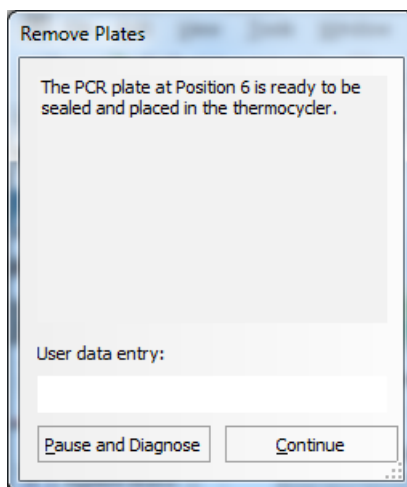
- 17 Verify that the Current Tip State indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See [page 23](#) for more information on using this segment of the form during the run.

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



19 Running the 03a WholeGenomePCR_QXT_ILM_v1.0.pro protocol takes approximately 10 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.

20 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.



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

22 Transfer the PCR plate to a thermal cycler and run the PCR amplification program shown in [Table 21](#).

3 Sample Preparation

Step 4. Amplify adaptor-ligated libraries

Table 21 Thermal cycler program for PCR indexing

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 5. Purify amplified DNA using AMPure XP beads

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

This step uses protocol **04a Cleanup_WholeGenomePCR_QXT_ILM_v1.0.pro**.

Prepare the NGS Bravo and reagents

- 1 Clear the Bravo deck of all plates and tip boxes, then wipe it down with a DNA Away decontamination wipe.
- 2 Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in [Table 12](#). See [page 17](#) for more information on how to do this step.

Table 22 Bravo Deck Temperature Presets

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

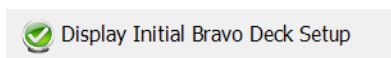
- 3 Verify that the AMPure XP bead suspension is at room temperature. (If necessary, allow the bead solution to come to room temperature for at least 30 minutes.) *Do not freeze the beads at any time.*
- 4 Mix the AMPure XP bead suspension well so that the reagent appears homogeneous and consistent in color.
- 5 Prepare a Nunc DeepWell source plate for the beads by adding 35 μ L of homogeneous AMPure XP beads per well, for each well to be processed.
- 6 Prepare a Thermo Scientific Matrix reservoir containing 15 mL of nuclease-free water.
- 7 Prepare a separate Thermo Scientific Matrix reservoir containing 45 mL of freshly-prepared 70% ethanol.
- 8 Centrifuge the amplified DNA sample plate for 30 seconds to drive the well contents off the walls and plate seal.

3 Sample Preparation

Step 5. Purify amplified DNA using AMPure XP beads

Setup and run VWorks 04a Cleanup_WholeGenomePCR_QXT_ILM_v1.0.pro

- 9 On the SureSelect setup form, under **Select Protocol**, select **04a Cleanup_WholeGenomePCR_QXT_ILM_v1.0.pro**.
- 10 Under **Select PCR plate Labware**, select the specific type of PCR plate used for amplification in the previous step.
- 11 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
- 12 Click **Display Initial Bravo Deck Setup**.



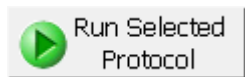
- 13 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in [Table 23](#).

Table 23 Initial Bravo deck configuration for 04a Cleanup_WholeGenomePCR_QXT_ILM_v1.0.pro

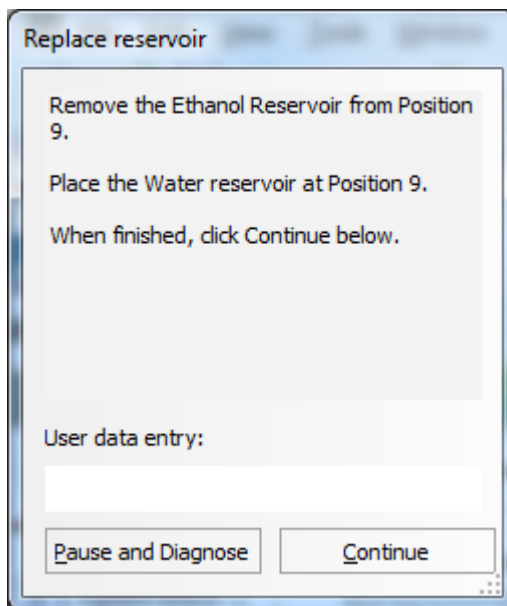
Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
2	New tip box
3	Empty Eppendorf plate
4	–(empty)–
5	AMPure XP beads in Nunc DeepWell plate (35 µL beads per processing well)
6	Amplified DNA libraries in unsealed PCR plate seated in red insert (PCR plate type must be specified on setup form under step 2)
7	–(empty)–
8	Empty tip box
9	70% ethanol in Matrix reservoir

- 14 Verify that the Current Tip State indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See [page 23](#) for more information on using this segment of the form during the run.

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



Running the 04a Cleanup_WholeGenomePCR_QXT_ILM_v1.0.pro protocol takes approximately 45 minutes. An operator must be present during the run to complete tip box replacement and to transfer the water reservoir to the Bravo deck, when directed by the VWorks prompt shown below.



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

3 Sample Preparation

Step 6. Assess library DNA quantity and quality using the 2100 Bioanalyzer and High Sensitivity DNA Assay

Step 6. 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. See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com for more information on doing this step.

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 5](#), 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 μ L of a 1:10 dilution of each sample for the analysis. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- 3 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 5](#).
 - 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.

- 4 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.

Step 6. Assess library DNA quantity and quality using the 2100 Bioanalyzer and High Sensitivity DNA 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.

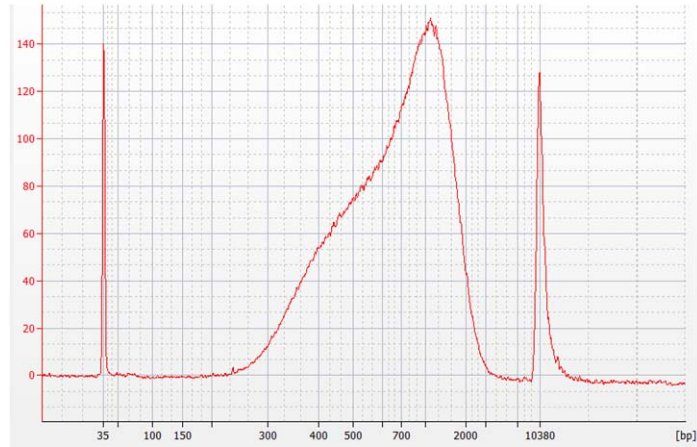


Figure 5 Analysis of amplified library DNA using a High-Sensitivity DNA Assay.

Step 7. 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 44](#).

- 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 24](#) 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 24 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.

Step 7. Pool samples for multiplexed sequencing

- 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 8. Prepare sequencing samples

The optimal seeding concentration for SureSelect^{QXT} whole genome libraries is 14 to 20 pM on HiSeq or MiSeq instruments and 1.2 to 1.4 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.

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. See [Table 25](#) for kit configurations compatible with the recommended read length plus reads for the SureSelect^{QXT} 8-bp dual indexes. To do this step, refer to the manufacturer's instructions, using the modifications described in [“Using the SureSelect^{QXT} Read Primers with Illumina's Paired-End Cluster Generation Kits”](#) on page 61.

Table 25 Illumina Kit Configuration Selection Guidelines

Platform	Run Type	Read Length [*]	SBS Kit Configuration	Chemistry
HiSeq 2500	Rapid Run	2 × 100 bp	200 Cycle Kit	v1 or v2
HiSeq 2500	High Output	2 × 100 bp	4 × 50 Cycle Kit [†]	v3
HiSeq 2500	High Output	2 × 125 bp	250 Cycle Kit	v4
HiSeq 2000	All Runs	2 × 100 bp	4 × 50 Cycle Kit [†]	v3
MiSeq	All Runs	2 × 150 bp	300 Cycle Kit	v2
MiSeq	All Runs	2 × 300 bp	600 Cycle Kit	v3
NextSeq 500	All Runs	2 × 150 bp	300 Cycle Kit	v2

* 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.

† A single 200-cycle kit does not include enough reagents to complete Reads 1 and 2 in addition to the dual 8-bp index reads in this format. If preferred, the additional reads may be supported by using one 200-cycle kit plus one 50-cycle kit.

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

To sequence the SureSelect^{QXT} libraries on Illumina 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 Read Primer
- SureSelect QXT Index 2 Read Primer NSQ (NextSeq platform only)

These SureSelect^{QXT} custom sequencing primers are provided at 100 μ M and must be diluted 1:200 in the corresponding Illumina primer solution, using the platform-specific instructions below:

For the HiSeq platform, combine the primers as shown in [Table 26](#) or [Table 27](#) on [page 62](#).

For the MiSeq platform, combine the primers as shown in [Table 28](#) on [page 62](#).

For the NextSeq platform, combine the primers as shown in [Table 29](#) or [Table 30](#) on [page 63](#).

NOTE

It is important to combine the primers precisely in the indicated ratios. Be sure to use measured volumes of each solution; do not use volumes reported on vial labels when preparing the mixtures. Vortex each mixture vigorously to ensure homogeneity for proper detection of the indexes using the custom read primers.

3 Sample Preparation

Step 8. Prepare sequencing samples

Table 26 HiSeq2000 and HiSeq 2500 High Output custom sequencing primer preparation

Sequencing Read	Volume of SureSelect QXT Primer	Volume of Illumina TruSeq Primer	Total Volume
Read 1	5 µL SureSelect QXT Read Primer 1 (brown cap)	995 µL HP6 or HP10	1 mL*
Index	15 µL SureSelect QXT Index Read Primer (clear cap)	2985 µL HP8 or HP12	3 mL
Read 2	15 µL SureSelect QXT Read Primer 2 (black cap)	2985 µL HP7 or HP11	3 mL

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

Table 27 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 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

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

Table 28 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 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 29 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 + Index 2	6 µl SureSelect QXT Index Read Primer (clear cap) + 6 µl SureSelect QXT Index 2 Read Primer NSQ (purple cap)	1988 µl BP14 (from well 22)	2 ml	well 9

Table 30 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 + Index 2	4.8 µl SureSelect QXT Index Read Primer (clear cap) + 4.8 µl SureSelect QXT Index 2 Read Primer NSQ (purple cap)	1590.4 µl BP14 (from well 22)	1.6 ml	well 9

3 Sample Preparation

Step 9. Set up the sequencing run and trim adaptors from the reads

Step 9. Set up the sequencing run and trim adaptors from the reads

Refer to Illumina protocols to set up custom sequencing primer runs, using the additional guidelines outlined below.

For SureSelect^{QXT} dual index sequence information, see tables on [page 42](#).

Before aligning reads to the reference genome, SureSelect^{QXT} adaptor sequences must be trimmed from the reads. You can use SureCall, Agilent's NGS data analysis software, to perform adaptor trimming, alignment of reads and variant calling of sequencing data generated from either the HiSeq or the MiSeq platform. To download SureCall free-of-charge and for additional information, including tutorials on this software, visit the [SureCall page at www.genomics.agilent.com](http://www.genomics.agilent.com).

SureCall is compatible with FASTQ files generated by both the HiSeq and MiSeq platforms. To use SureCall to analyze SureSelect^{QXT}-generated data, you first need to define an analysis workflow. This analysis workflow identifies the libraries as SureSelect^{QXT} libraries and enables automated adaptor trimming. The trimmed FASTQ files can then be used for alignment to generate BAMs for downstream analysis.

To create the analysis workflow, refer to [Figure 6 on page 65](#). Upon starting SureCall, click the **Analysis Workflow** tab. Choose the appropriate analysis type (single sample, paired, or trio analysis), and then click the **Import Unaligned Files** button. Within the *Select Unaligned Sample Files* window, specify your read 1 and read 2 files using the **Add** buttons. Using the menus near the bottom of the screen, select **Default SureSelect QXT Method** from the *Analysis Method* menu, choose the appropriate design description from the *Design* menu, and select **Illumina** from the *Platform* menu. Once done, refer to the SureCall guide for next steps on alignment and variant calling.

If using another pipeline for alignment and downstream analysis, refer to the platform-specific guidelines starting on [page 65](#).

Step 9. Set up the sequencing run and trim adaptors from the reads

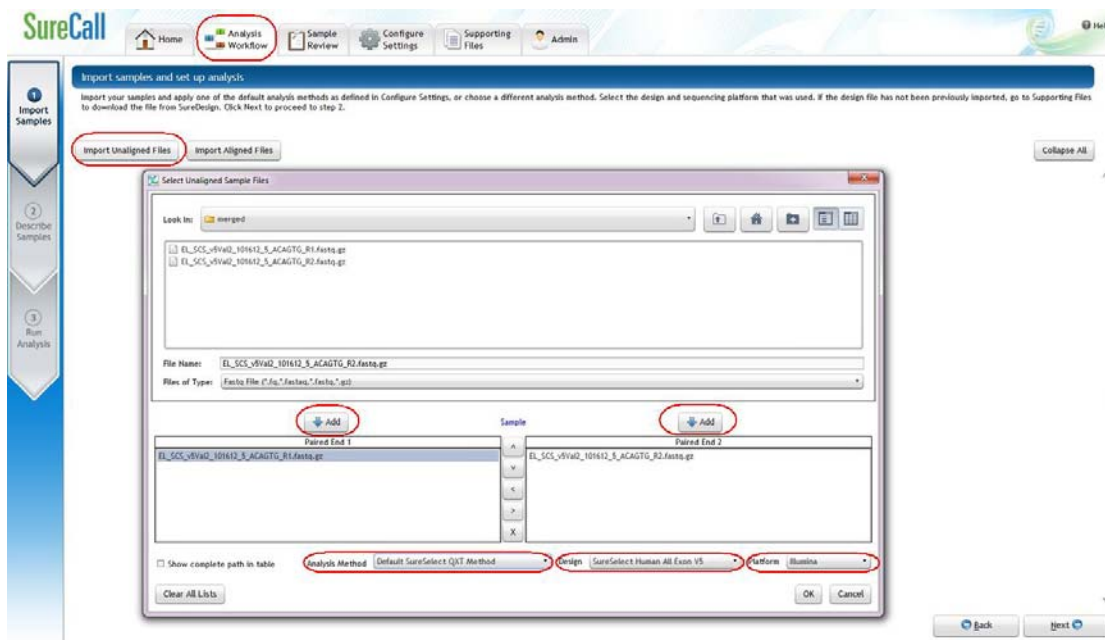


Figure 6 Analysis workflow setup in SureCall.

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

HiSeq or NextSeq 500 platform sequencing run setup and adaptor trimming guidelines

Set up sequencing runs using the *Custom* setting. 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.

3 Sample Preparation

Step 9. Set up the sequencing run and trim adaptors from the reads

Use the *Cycles* settings shown in [Table 31](#). 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 31 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 Agilent's Read Trimmer tool. This tool takes in data in FASTQ format and removes the adaptor sequence from the ends of the sequencing reads, generating trimmed FASTQ data as output. To download the Read Trimmer tool free-of-charge and for additional information on this resource, visit www.agilent.com/genomics/AgilentReadTrimmer.



4 Reference

Kit Contents	68
Nucleotide Sequences of SureSelect ^{OXT} Dual Indexes	70
Guidelines for Multiplexing with Dual-Indexed Samples	72

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 32 SureSelect^{QXT} Library Prep Kit Contents

Component Kits	Storage Condition	HiSeq or MiSeq (ILM) 96 Samples	NextSeq (NSQ) 96 Samples
SureSelect QXT Library Prep Kit, ILM, Box 1	Room Temperature	5500-0119	5500-0119
SureSelect QXT Library Prep Kit Box 2	-20°C	5500-0121	5500-0127

The contents of each of the component kits listed in [Table 32](#) are described in [Table 33](#) through [Table 35](#) below.

Table 33 SureSelect QXT Library Prep, ILM, Box 1 Content

Kit Component	96 Reactions
SureSelect QXT Stop Solution	bottle

Table 34 SureSelect QXT Library Prep, ILM, Box 2 Content

Kit Component	HiSeq or MiSeq (ILM) 96 Reactions
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 Read Primer	tube with clear 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)

Table 35 SureSelect QXT Library Prep, NSQ, Box 2 Content

Kit Component	NextSeq (NSQ) 96 Reactions
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 Read Primer	tube with clear cap
SureSelect QXT Index 2 Read Primer NSQ	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 number 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 36 SureSelect^{QXT} P7 Indexes 1 to 12

Index 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 37 SureSelect^{OXT} P5 Indexes 13 to 20 for HiSeq platform, MiSeq platform, or NextSeq platform runs through BaseSpace

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 38 SureSelect^{OXT} P5 Indexes 13 to 20 for NextSeq 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 NextSeq runs through BaseSpace, use the reverse complement sequences provided in [Table 37](#).

4 Reference

Guidelines for Multiplexing with Dual-Indexed Samples

Guidelines for Multiplexing with Dual-Indexed Samples

Agilent recommends following the dual index sample pooling guidelines and shown in [Table 39](#). 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 also be allowed during demultiplexing in order to maximize sequencing output per sample.

Table 39 Dual index sample pooling guidelines for 96 Reaction Kits

Plexity of Sample Pool	Recommended SureSelect ^{OXT} P7 Indexes	Recommended SureSelect ^{OXT} P5 Indexes
1-plex	Any P7 index i1 to i11	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 11-plex	Any combination of P7 indexes i1 to i11 using each index only once	P5 i13 and P5 i14 OR P5 i15 and P5 i16 OR P5 i17 and P5 i18 (as needed)
12- to 88-plex	Any combination of P7 indexes i1 to i11 (as needed)	P5 i13 and P5 i14 and any third P5 index OR P5 i15 and P5 i16 and any third P5 index OR P5 i17 and P5 i18 and any third P5 index (as needed)
89- to 96-plex	All twelve P7 indexes (i1 to i12)	Any P5 indexes (i13 to i20, as needed)

* The indicated indexes may be substituted with another index within range of P7 i1 to P7 i11, as long as the substitute index differs from all others used in the sample pool.

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In This Book

This guide contains information to run the SureSelect^{QXT} Automated Whole Genome Library Prep protocol using Agilent's NGS Bravo Option A.

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