SureSelect^{QXT} Automated Target Enrichment 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.

Agilent Technologies

Notices

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Acknowledgement

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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 Target Enrichment system.

This protocol is specifically developed and optimized to enrich targeted regions of the genome from repetitive sequences and sequences unrelated to the research focus prior to sample sequencing. Sample processing steps are automated using 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} Target Enrichment

This chapter contains an orientation to the Agilent NGS Bravo, an overview of the SureSelect target enrichment 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 target enrichment.

4 Hybridization

This chapter describes the steps to hybridize and capture the prepared DNA library using a SureSelect or ClearSeq Capture Library.

5 Indexing

This chapter describes the steps for post-capture amplification and guidelines for sequencing sample preparation.

6 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 (seeTable 61 and Table 62 on page 114)
- Update to P5 Index details for NextSeq 500 platform runs using BaseSpace (see Table 70 on page 124)
- Update to Qubit dsDNA Assay Kit nomenclature (Table 1 on page 12 and step 4 on page 39)
- Updates to SureCycler 8800 PCR plate compatibility considerations (see *Caution* on page 34)
- Support for Agilent 4200 TapeStation (see Table 4 on page 16)
- Correction to ordering information for Axygen 96 Deep Well plates (see Table 4 on page 15)
- Revised ordering information for nucleic acid surface decontamination wipes (Table 4 on page 15)

What's New in Version A1

- Support for ClearSeq Capture Libraries, including ClearSeq Comprehensive Cancer Libraries (see Table 3 on page 14).
- Support for Human All Exon v6 Capture Libraries (see Table 2 on page 13).
- Update to SBS Kit Configuration details for HiSeq 2500 Rapid Run sequencing (see Table 57 on page 111).
- Support for sequencing using NextSeq 500 v2 (see Table 61 on page 111).
- Update to Qubit dsDNA Assay Kit ordering information (see Table 1 on page 12).

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 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 G9681-90010. For non-automated sample processing procedures see publication G9681-90000.

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

Procedural Notes

- 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 39. 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.

Required Reagents

Table 1 Required Reagents for SureSelect^{QXT} Target Enrichment

Description	Vendor and part number
SureSelect or ClearSeq Capture Library	Select one library from Table 2 or Table 3
SureSelect^{QXT} Reagent Kit Illumina HiSeq or MiSeq platform (ILM), 96 Samples Illumina NextSeq platform (NSQ), 96 Samples	Agilent p/n G9681B p/n G9683B
Agencourt AMPure XP Kit 5 ml 60 ml 450 ml	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
Dynabeads MyOne Streptavidin T1 2 ml 10 ml 100 ml	Life Technologies p/n 65601 p/n 65602 p/n 65603
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 *or*	Life Technologies p/n Q32851
Qubit dsDNA BR Assay Kit 100 assays 500 assays	Life Technologies p/n Q32850 p/n Q32853
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930

Table 2 SureSelect^{XT} Automation Capture Libraries

Capture Library	96 Reactions	480 Reactions
SureSelect^{XT} Human All Exon v6	5190-8865	5 × 5190-8865
SureSelect^{XT} Human All Exon v6 + UTRs	5190-8883	5 × 5190-8883
SureSelect^{XT} Human All Exon v6 + COSMIC	5190-9309	5 × 5190-9309
SureSelect^{XT} Human All Exon v6 Plus 1	5190-8868	5 × 5190-8868
SureSelect^{XT} Human All Exon v6 Plus 2	5190-8871	5 × 5190-8871
SureSelect^{XT} Clinical Research Exome	5190-7344	5 × 5190-7344
SureSelect^{XT} Focused Exome	5190-7789	5 × 5190-7789
SureSelect^{XT} Focused Exome Plus 1	5190-7792	5 × 5190-7792
SureSelect^{XT} Focused Exome Plus 2	5190-7796	5 × 5190-7796
SureSelect^{XT} Human All Exon v5	5190-6210	5 × 5190-6210
SureSelect^{XT} Human All Exon v5 + UTRs	5190-6215	5 × 5190-6215
SureSelect^{XT} Human All Exon v5 + lncRNA	5190-6448	5 × 5190-6448
SureSelect^{XT} Human All Exon v5 Plus	5190-6224	5 × 5190-6224
SureSelect^{XT} Human All Exon v4	5190-4633	5190-4635
SureSelect^{XT} Human All Exon v4 + UTRs	5190-4638	5190-4640
SureSelect^{XT} Mouse All Exon	5190-4643	5190-4645
SureSelect^{XT} Custom 1 kb up to 499 kb (reorder)	5190-4808 (5190-4813)	5190-4810 (5190-4815)
SureSelect^{XT} Custom 0.5 Mb up to 2.9 Mb (reorder)	5190-4818 (5190-4823)	5190-4820 (5190-4825)
SureSelect^{XT} Custom 3 Mb up to 5.9 Mb (reorder)	5190-4828 (5190-4833)	5190-4830 (5190-4835)
SureSelect^{XT} Custom 6 Mb up to 11.9 Mb (reorder)	5190-4838 (5190-4843)	5190-4840 (5190-4845)
SureSelect^{XT} Custom 12 Mb up to 24 Mb (reorder)	5190-4898 (5190-4903)	5190-4900 (5190-4905)

Table 3 Compatible ClearSeq Automation Capture Libraries

Capture Library	96 Reactions	480 Reactions
ClearSeq Comprehensive Cancer XT	5190-8013	5 × 5190-8013
ClearSeq Comprehensive Cancer Plus XT	5190-8016	5 × 5190-8016
ClearSeq Inherited Disease XT	5190-7520	5 × 5190-7520
ClearSeq Inherited Disease Plus XT	5190-7523	5 × 5190-7523
ClearSeq DNA Kinome XT	5190-4648	5190-4650

Optional Reagents

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

Required Equipment

Table 4 Required Equipment for SureSelect^{QXT} Target Enrichment

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 8 on page 35 for the list of PCR plates supported in automation protocols	Agilent p/n 401334
Eppendorf twin.tec full-skirted 96-well PCR plates	Eppendorf p/n 951020401 or 951020619
Thermo Scientific Reservoirs	Thermo Scientific p/n 1064156
Nunc DeepWell Plates, sterile, 1.3-ml well volume	Thermo Scientific p/n 260251
Axygen 96 Deep Well Plate, 2 mL, Square Well (waste reservoirs; working volume 2.2 mL)	Axygen p/n P-2ML-SQ-C E & K Scientific p/n EK-2440
DNA LoBind Tubes, 1.5-ml PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
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
Magnetic separator	DynaMag-50 magnet, Life Technologies p/n 123-02D or equivalent

Table 4 Required Equipment for SureSelect^{QXT} Target Enrichment

Description	Vendor and part number
Vacuum concentrator	Savant SpeedVac, model DNA120, with 96-well plate rotor, model RD2MP, or equivalent
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
OR	
Agilent 4200 TapeStation	Agilent p/n G2991AA
Agilent D1000 ScreenTape	Agilent p/n 5067-5582
Agilent D1000 Reagents	Agilent p/n 5067-5583
Agilent High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
Agilent D1000 Reagents	Agilent p/n 5067-5585
OR	
Agilent 2200 TapeStation	Agilent p/n G2964AA or G2965AA
Agilent D1000 ScreenTape	Agilent p/n 5067-5582
Agilent D1000 Reagents	Agilent p/n 5067-5583
Agilent High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
Agilent D1000 Reagents	Agilent p/n 5067-5585
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
Pipettes (multichannel pipette and P10, P20, P200 and P1000 pipettes)	Pipetman 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} Target Enrichment

This chapter contains an orientation to the Agilent NGS Bravo Option A, an overview of the SureSelect^{QXT} target enrichment 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 µl to 250 µl.

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

Bravo Platform Deck

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

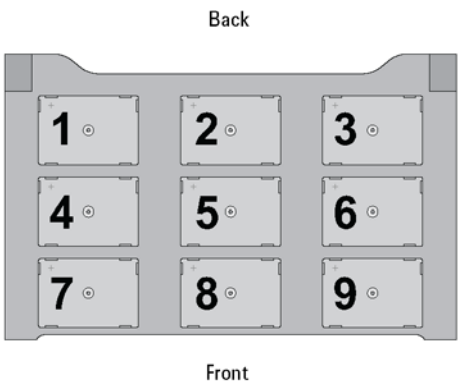


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

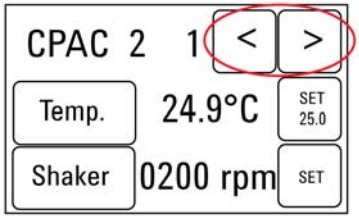
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

Bravo deck heat block temperatures may be changed using the Inheco Multi TEC Control device touchscreen as described in the steps below. See Table 5 for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

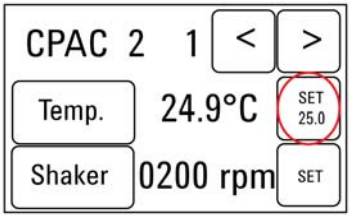
Table 5 Inheco Multi TEC Control touchscreen designations

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

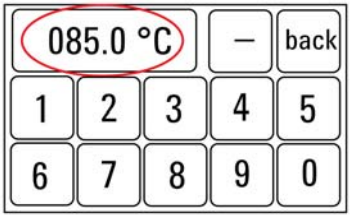
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



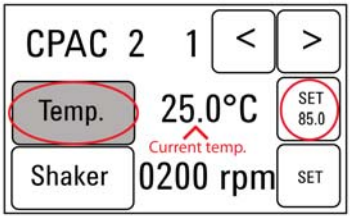
2 To set the temperature of the selected block, press the SET button.



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



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



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

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

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

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

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

3 Press the **START** button.

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

VWorks Automation Control Software

VWorks software, included with your Agilent NGS 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^{QXT} automation protocols version 1.0.

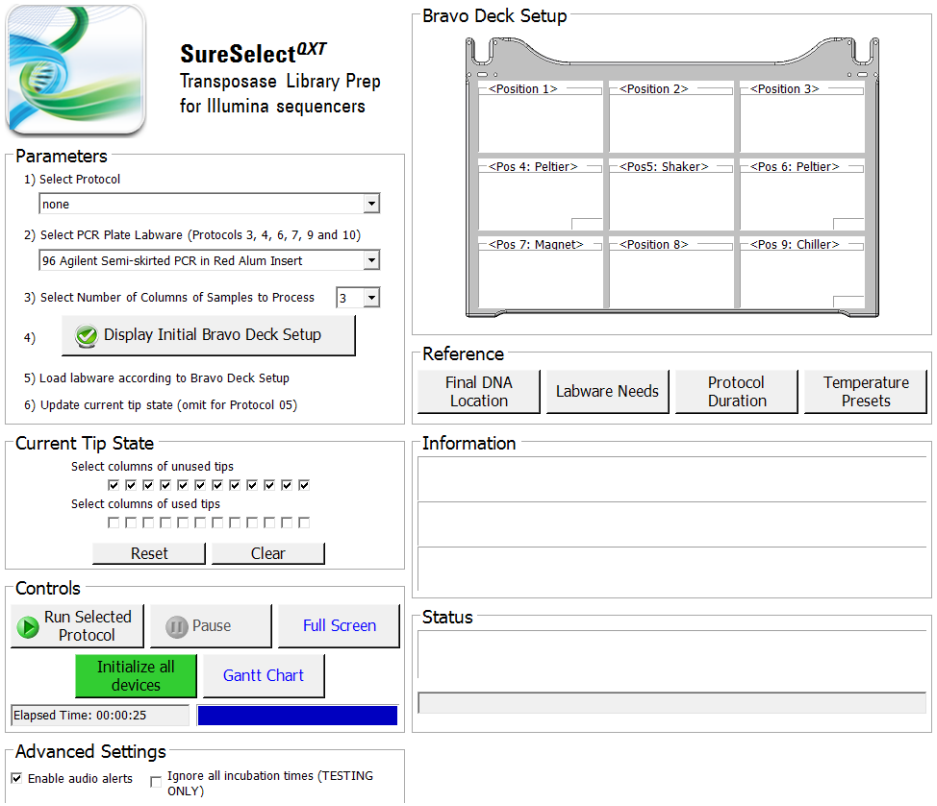
If you have questions about VWorks version compatibility, please contact service.automation@agilent.com.

Logging in to the VWorks software

1. Double-click the VWorks icon or the 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.)

Using the QXT_ILM_OptA_v1.0.VWForm to setup and start a run

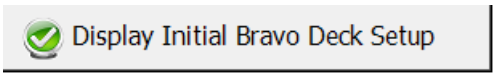
Use the VWorks form QXT_ILM_OptA_v1.0.VWForm, shown below, to set up and start each SureSelect automation protocol.



1 Open the form using the QXT_ILM_OptA_v1.0.VWForm shortcut on your desktop.

2 Use the drop-down menus on the form to select the appropriate SureSelect workflow step and other Parameters for the run.

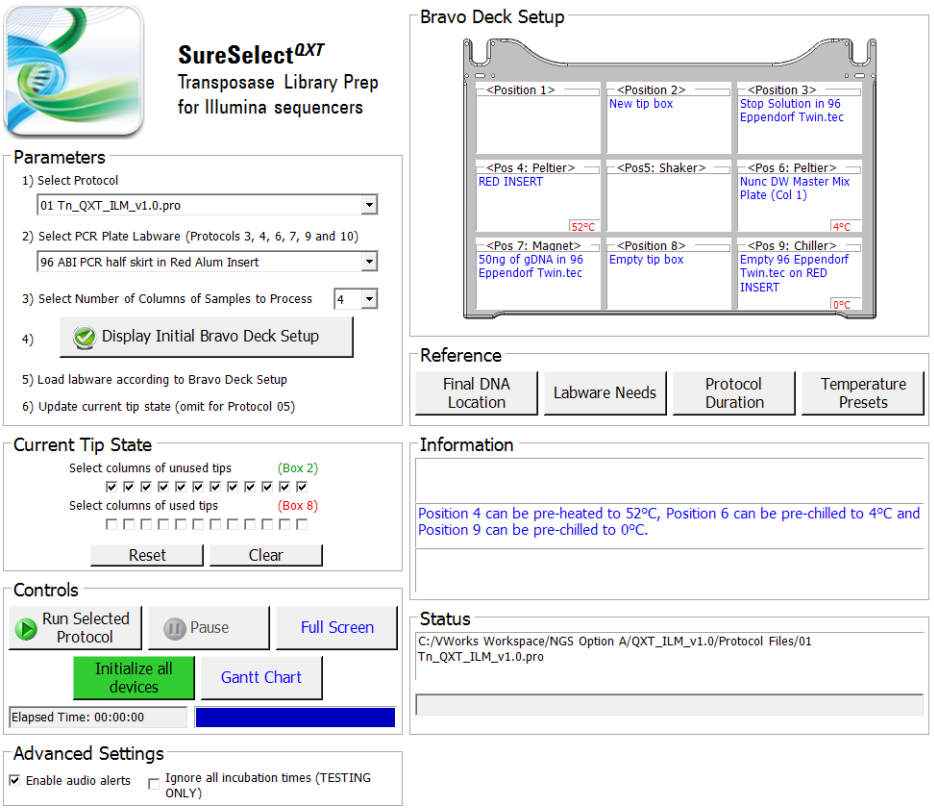
3 Once all run parameters have been specified on the form, click **Display Initial Bravo Deck Setup.**



4 The form will then display the NGS Bravo deck configuration needed for the specified run parameters.

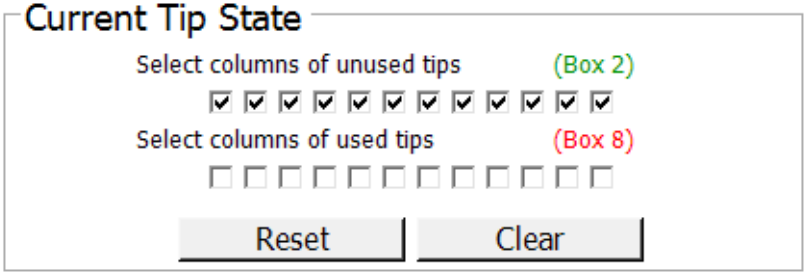
Load the Bravo Deck with labware and reagents as specified in the **Bravo Deck Setup** region of the form.

Review the temperature preset and in-run labware transfer information shown in the **Information** section of the form. Set the temperature of Bravo Deck positions as needed.



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.



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.

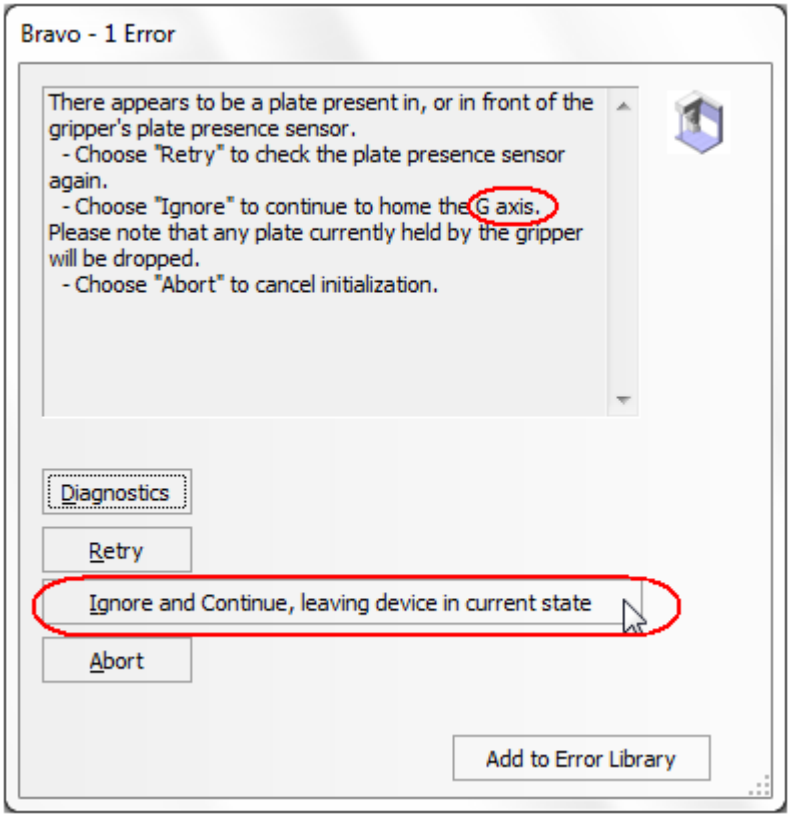
You can use partial tip boxes for NGS Bravo Option A automation protocols, as long as positions of available tips are accurately indicated during run setup.

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

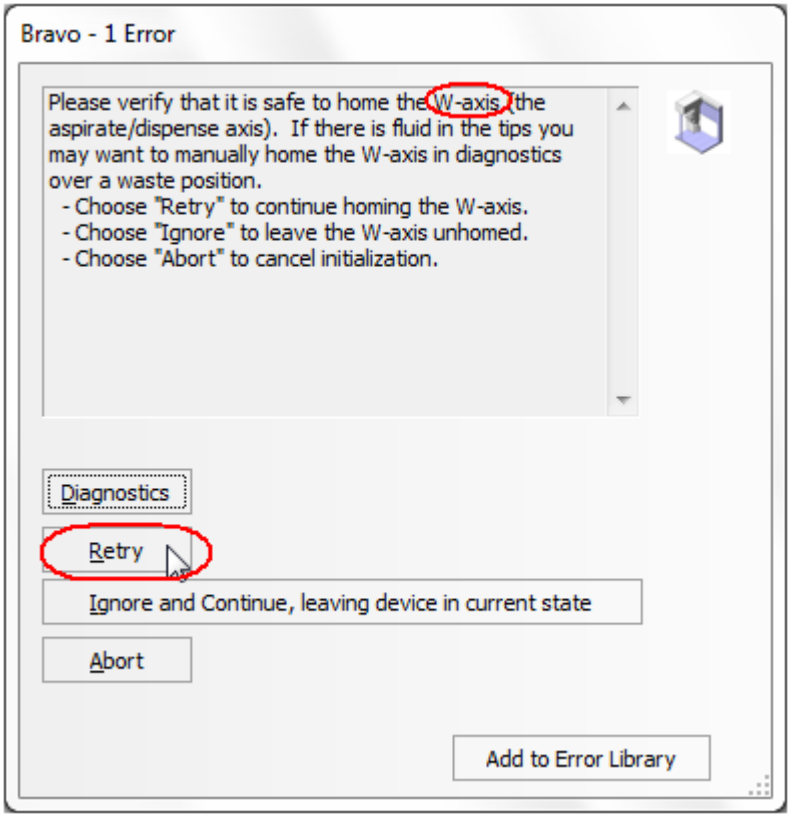
Error messages encountered at start of run

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} Target Enrichment Procedure

Figure 2 summarizes the SureSelect^{QXT} target enrichment workflow for samples to be sequenced using the Illumina paired-read sequencing platform. For each sample to be sequenced, individual library preparations, hybridizations, and captures are performed. The samples are then tagged by PCR with an index sequence. Depending on the target size of the SureSelect capture, 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 6 summarizes how the VWorks protocols are integrated into the SureSelect^{QXT} workflow. See Sample Preparation, Hybridization, and Indexing chapters for complete instructions for use of the VWorks protocols for sample processing.

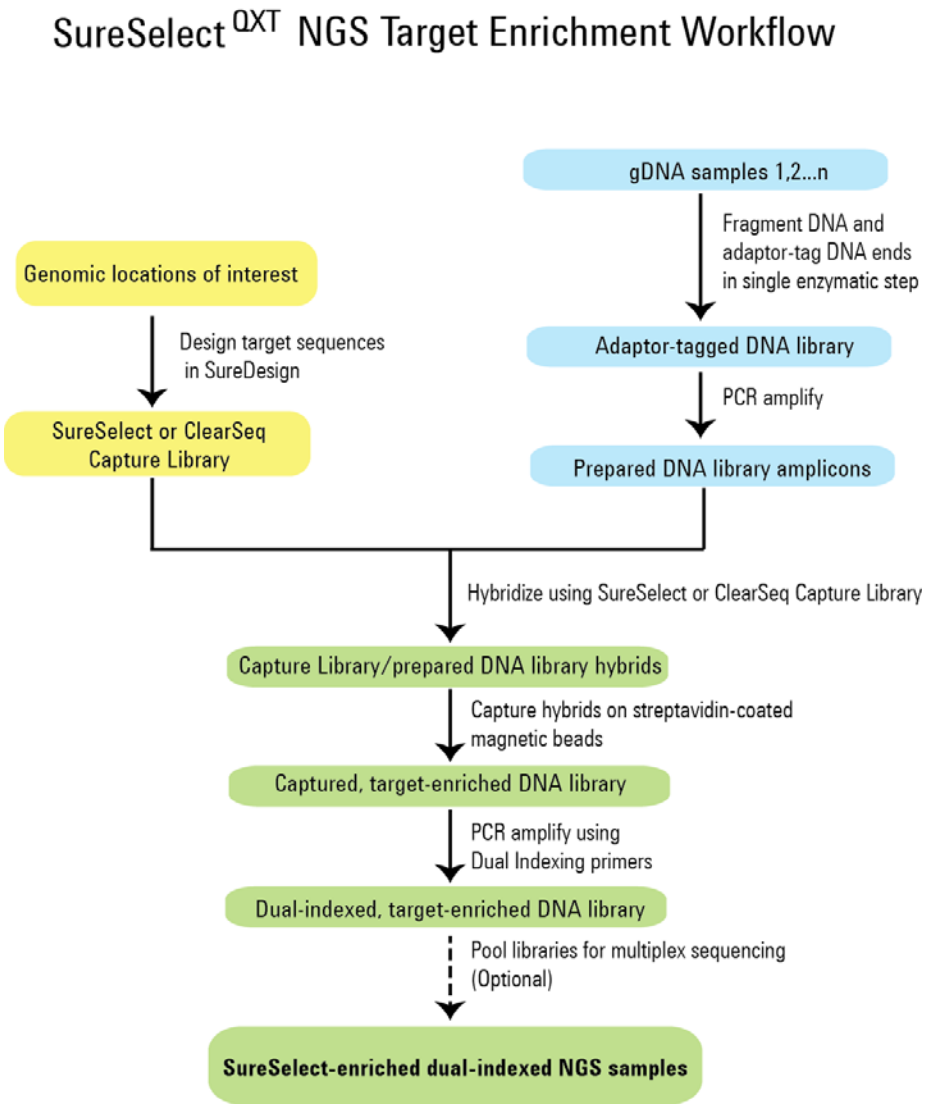


Figure 2 Overall sequencing sample preparation workflow.

Table 6 Overview of VWorks protocols used for SureSelect^{QXT} Target Enrichment using NGS Bravo Option A

Workflow Step (Protocol Chapter)	Substep	VWorks Protocols Used for Agilent NGS Bravo Option A automation
Sample Preparation	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	03b Pre-CapturePCR_QXT_ILM_v1.0.pro
	Purify DNA using AMPure XP beads	04b Cleanup_Pre-CapturePCR_QXT_ILM_v1.0.pro
Hybridization	Aliquot prepped libraries for hybridization	05 Aliquot_Libraries_v1.0.pro
	Hybridize prepped DNA to Capture Library	06 Hybridization_QXT_v1.0.pro
	Capture DNA hybrids	07 SureSelectQXT_Capture_v1.0.pro
	Wash captured DNA hybrids	08 SureSelectQXT_Wash_v1.0.pro
Indexing	Add index tags by PCR	09 Post-CapturePCR_QXT_ILM_v1.0.pro
	Purify DNA using AMPure XP beads	10 Cleanup_Post-CapturePCR_QXT_ILM_v1.0.pro

Experimental Setup Considerations for Automated Runs

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

Table 7 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.
- At the hybridization step (see Figure 2), you can add a different SureSelect or ClearSeq Capture Library to each row of the plate. Plan your experiment such that each prepared DNA library plate position corresponds to the appropriate Capture Library.
- For post-capture amplification (see Figure 2), different SureSelect or ClearSeq Capture Libraries can require different amplification cycle numbers, based on sizes of the captured targets. It is most efficient to process similar-sized Capture Libraries on the same plate. See Table 53 on page 101 to determine which Capture Libraries may be amplified on the same plate.
- Post-capture 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 after hybridization to the Capture Library (see Figure 2). It is important to review and understand the guidelines for assignment of dual indexing primers on page 96 while planning sample placement for the run to ensure that the indexing design is compatible with the initial DNA sample placement.

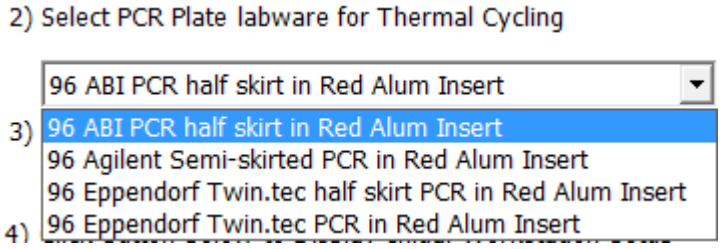
Considerations for Equipment Setup

- 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 8.



CAUTION

The plates listed in Table 8 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 8 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 8.

Table 8 Ordering information for supported PCR plates

Description in VWorks menu	Vendor and part number
96 ABI PCR half-skirted plates (MicroAmp Optical plates)	Life Technologies p/n N8010560
96 Agilent semi-skirted PCR plate	Agilent p/n 401334
96 Eppendorf Twin.tec half-skirted PCR plates	Eppendorf p/n 951020303
96 Eppendorf Twin.tec PCR plates (full-skirted)	Eppendorf p/n 951020401 or 951020619

3 Sample Preparation

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

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 50.
2. Prepare reagents for the purification protocols on page 45 and page 55.
   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 150 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 Hyb Module 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 41.

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 NGS Bravo, in well order A1 to H1, then A2 to H2, ending with A12 to H12.

NOTE

SureSelect Automated Library Prep and Capture System runs may include 1, 2, 3, 4, 6, or 12 columns of the plate. See Experimental Setup Considerations for Automated Runs on page 32 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 43.

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 9 in addition to reagents prepared for use on page 38 to page 39.

Table 9 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 40
SureSelect QXT Enzyme Mix ILM	SureSelect QXT Library Prep Kit Box 2, –20°C	page 40

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 10. See page 19 to page 21 for more information on how to do this step.

Table 10 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.

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 11. Mix well by vortexing for 20 seconds and then keep on ice.

Table 11 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
SureSelect QXT Enzyme Mix ILM	2.0 µL	25.5 µl	42.5 µl	59.5 µl	76.5 µl	110.5 µl	221.0 µl
Total Volume	**19 µl**	**242.3 µl**	**403.8 µl**	**565.3 µl**	**726.8 µl**	**1049.8 µl**	**2099.5 µl**

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 12 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 12 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 µl	48.1 µl	68.3 µl	88.5 µl	128.8 µl	260.1 µl

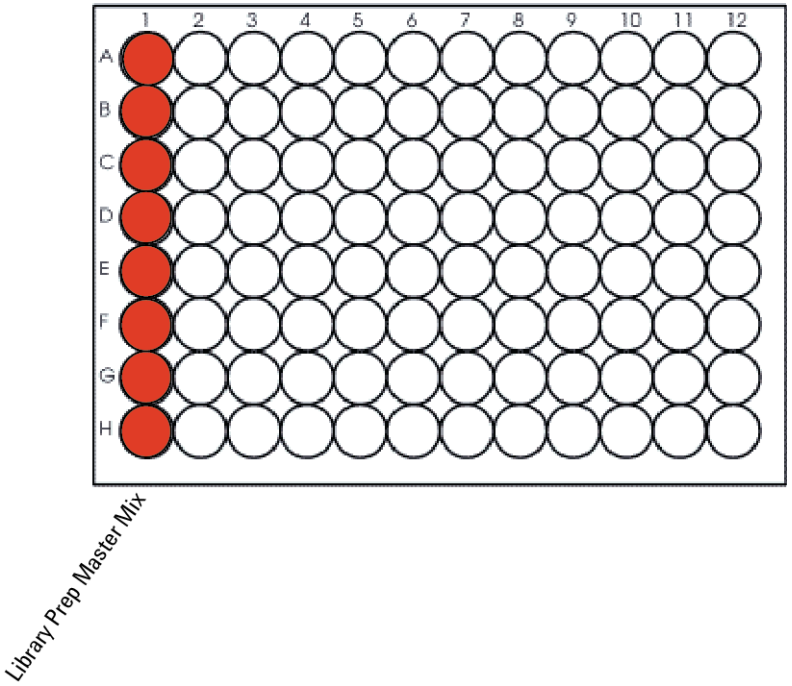


Figure 3 Configuration of the master mix source plate for 01 Tn_QXT_ILM_v1.0.pro

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

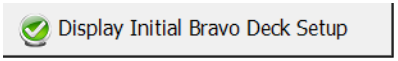
The presence of bubbles in source plate solutions may cause inaccurate volume transfer by the Bravo liquid handling platform. Ensure that the source plate is sealed and centrifuged prior to use in a run.

Setup and run VWorks protocol 01 Tn_QXT_ILM_v1.0.pro

10 On the SureSelect setup form, under **Select Protocol to Run**, 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 13.

Table 13 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

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 14. See page 19 for more information on how to do this step.

Table 14 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 µl 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.

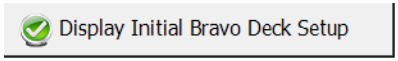
6 Prepare a separate Thermo Scientific Matrix reservoir containing 45 ml of freshly-prepared 70% ethanol.

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.

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 15.

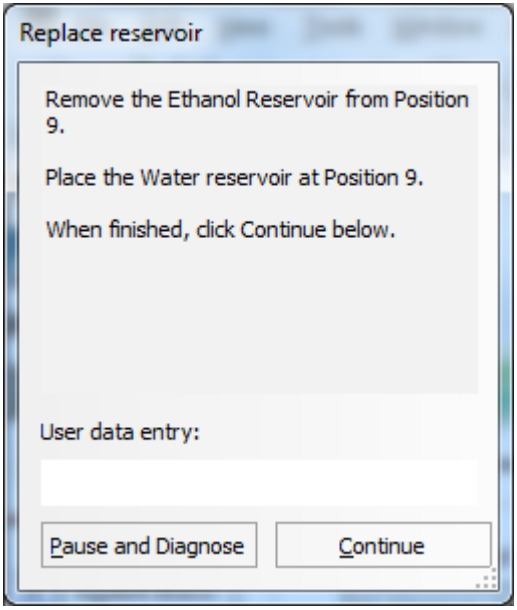
Table 15 Initial Bravo deck configuration for 02 Cleanup_Tn_v1.0.pro

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**.

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.

Step 4. Amplify adaptor-ligated libraries

In this step, the Agilent NGS Bravo completes the liquid handling steps for amplification of the adaptor-ligated DNA samples using automation protocol 03b Pre-CapturePCR_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 16.

Table 16 Reagents for precapture amplification

Kit Component	Storage Location	Where Used
Herculase II Fusion DNA Polymerase	SureSelect QXT Library Prep Kit Box 2, –20°C	page 49
Herculase II 5× Reaction Buffer	SureSelect QXT Library Prep Kit Box 2, –20°C	page 49
100 mM dNTP Mix (25 mM each dNTP)	SureSelect QXT Library Prep Kit Box 2, –20°C	page 49
SureSelect QXT Primer Mix	SureSelect QXT Hyb Module Box 2, –20°C	page 49
DMSO	Transferred to Room Temperature storage on page 38	page 49

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 17. See page 19 to page 21 for more information on how to do this step.

Table 17 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

3 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 pre-capture PCR master mix and master mix source plate

4 Prepare the appropriate volume of pre-capture PCR Master Mix, according to Table 18. Mix well using a vortex mixer and keep on ice.

Table 18 Preparation of Pre-Capture 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	13.5 µl	172.1 µl	286.9 µl	401.6 µl	516.4 µl	745.9 µl	1491.8 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
SureSelect QXT Primer Mix	1.0 µL	12.8 µl	21.3 µl	29.8 µl	38.3 µl	55.3 µl	110.5 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	**28.5 µl**	**363.4 µl**	**605.6 µl**	**847.9 µl**	**1090.1 µl**	**1574.6 µl**	**3149.3 µl**

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 19 to all wells of column 2 of the master mix source plate. The final configuration of the master mix source plate is shown in Figure 4.

Table 19 Preparation of the Master Mix Source Plate for 03b Pre-CapturePCR_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
Pre-Capture PCR Master Mix	Column 2 (A2-H2)	41.9 µl	72.1 µl	102.4 µl	132.7 µl	193.3 µl	390.1 µl

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

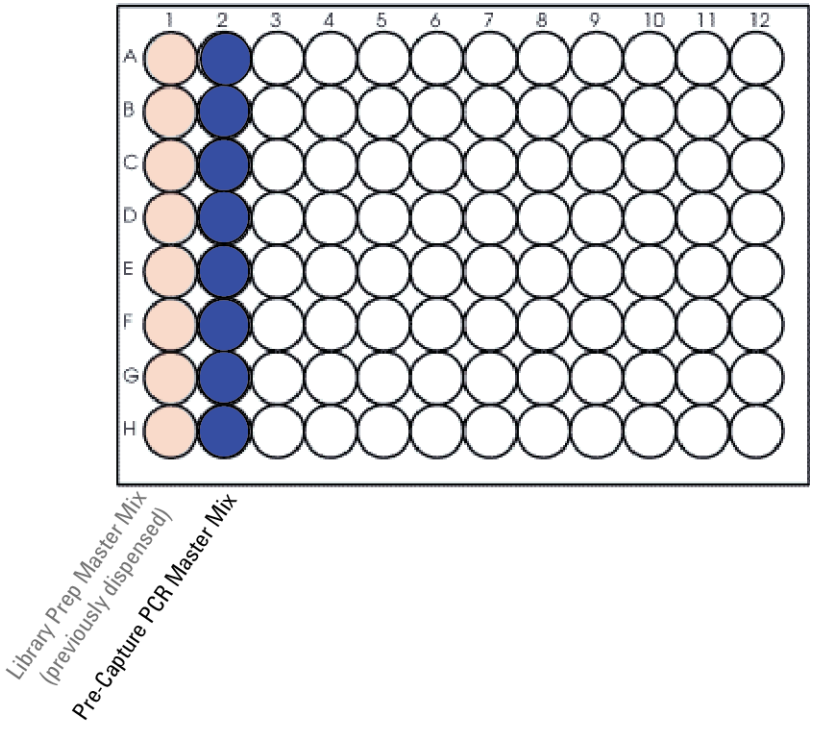


Figure 4 Configuration of the master mix source plate for 03b Pre-CapturePCR_QXT_ILM_v1.0.pro. Column 1 was used to dispense master mix during the previous protocol.

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

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

NOTE

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

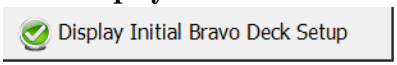
Setup and run VWorks protocol 03b Pre-CapturePCR_QXT_ILM_v1.0.pro

8 On the SureSelect setup form, under **Select Protocol to Run**, select **03b Pre-CapturePCR_QXT_ILM_v1.0.pro.**

9 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).

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

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



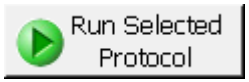
12 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 20.

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	Empty 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 PCR Master Mix in Column 2 (unsealed), seated in silver insert

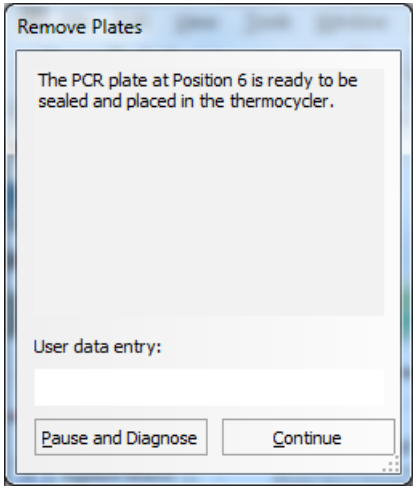
13 Verify that the Current Tip State indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 23 for more information on using this segment of the form during the run.

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



15 Running the 03b Pre-CapturePCR_QXT_ILM_v1.0.pro protocol takes approximately 10 minutes. Once complete, the PCR-ready samples, containing prepped DNA and PCR master mix, are located in the PCR plate at position 6 of the Bravo deck.

16 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.



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

18 Transfer the PCR plate to a thermal cycler and run the PCR amplification program shown in Table 21.

Table 21 Pre-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	68°C	2 minutes
2	1	98°C	2 minutes
3	8	98°C	30 seconds
		57°C	30 seconds
		72°C	1 minute
4	1	72°C	5 minutes
5	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 adaptor-tagged DNA to a Nunc DeepWell plate and then collects and washes the bead-bound DNA.

This step uses protocol **04b Cleanup_Pre-CapturePCR_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 14. See page 19 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 50 µl 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.

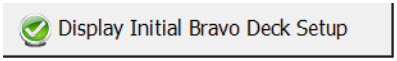
Setup and run VWorks 04b Cleanup_Pre-CapturePCR_QXT_ILM_v1.0.pro

9 On the SureSelect setup form, under **Select Protocol**, select **04b Cleanup_Pre-CapturePCR_QXT_ILM_v1.0.pro.**

10 Under **Select PCR plate Labware**, select the specific type of PCR plate used for pre-capture amplification.

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**.



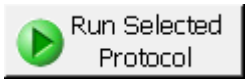
13 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 15.

Table 23 Initial Bravo deck configuration for 04b Cleanup_Pre-CapturePCR_QXT_ILM_v1.0.pro

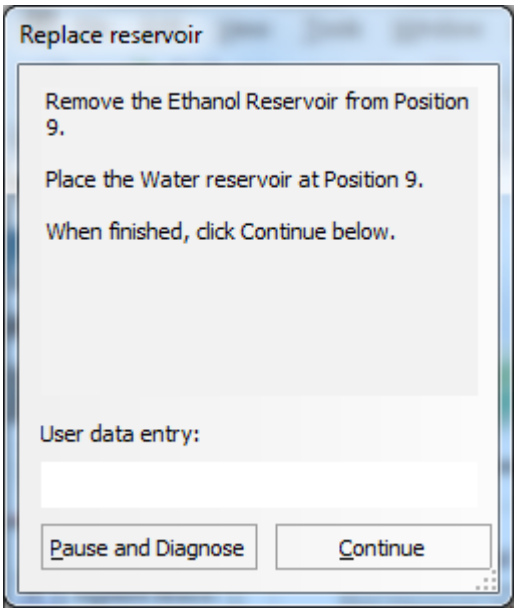
Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
2	New tip box
3	–(empty)–
4	–(empty)–
5	AMPure XP beads in Nunc DeepWell plate (50 µl beads per processing well)
6	Amplified DNA libraries in unsealed PCR plate seated in red insert (PCR plate type must be specified on 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 04b Cleanup_Pre-CapturePCR_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.

Step 6. Assess Library DNA quantity and quality

Measure the concentration of each library using one of the methods detailed below.

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

Use a Bioanalyzer DNA 1000 chip and reagent kit to analyze the amplified libraries. For more information to do this step, see the *Agilent DNA 1000 Kit Guide* at www.genomics.agilent.com.

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

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

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

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

5 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.

6 Verify that the electropherogram shows the peak of DNA fragment size positioned between 245 to 325 bp. Sample electropherograms are shown in Figure 5. Variability of fragmentation profiles may be observed.

NOTE

A peak DNA fragment size significantly less than 245 bp may indicate too little gDNA in the fragmentation reaction and may be associated with increased duplicates in the sequencing data. In contrast, a peak DNA fragment size significantly greater than 325 bp may indicate too much gDNA in the fragmentation reaction and may be associated with decreased percent-on-target performance in sequencing results.

7 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.

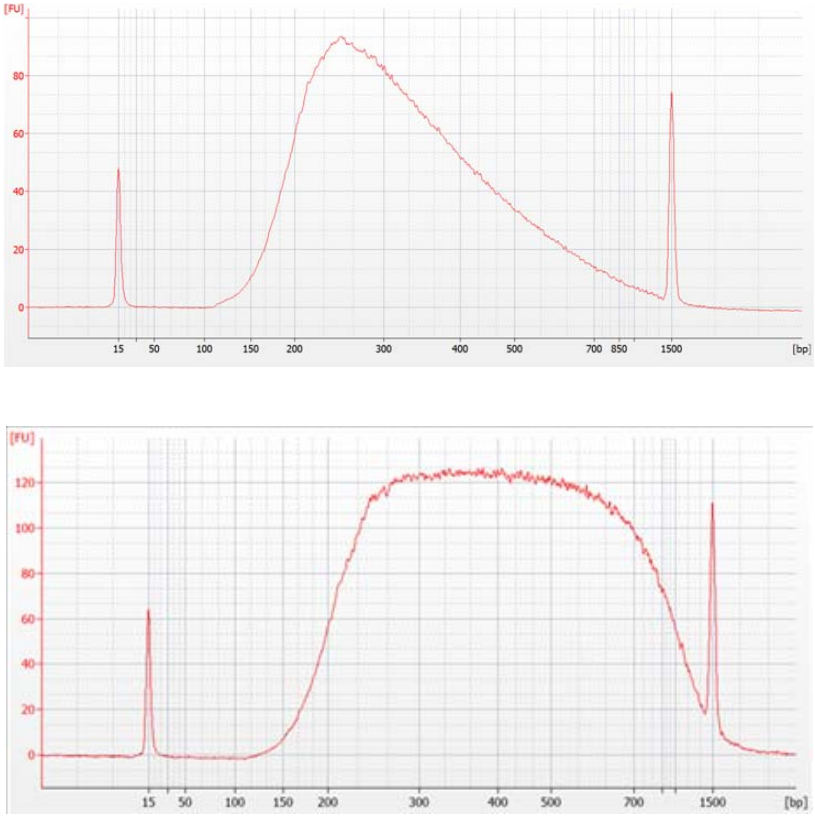


Figure 5 Representative sample electropherograms showing pre-capture analysis of amplified library DNA using the Agilent 2100 Bioanalyzer and a DNA 1000 Assay.

Option 2: Analysis using an Agilent TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape (p/n 5067-5582) and associated reagent kit (p/n 5067-5583) to analyze the amplified libraries using the Agilent 4200 TapeStation or 2200 TapeStation. For more information to do this step, see the appropriate TapeStation user manual at www.genomics.agilent.com.

1 Prepare the TapeStation samples as instructed in the instrument user manual. Use 1 µl of each amplified library DNA sample diluted with 3 µl of D1000 sample buffer for the analysis.

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

2 Load the sample plate or tube strips from step 1, the D1000 ScreenTape, and loading tips into the TapeStation as instructed in the instrument user manual. Start the run.

3 Verify that the electropherogram shows the peak of DNA fragment size positioned between 245 to 325 bp. Sample electropherograms are shown in Figure 6. Variability of fragmentation profiles may be observed.

NOTE A peak DNA fragment size significantly less than 245 bp may indicate too little gDNA in the fragmentation reaction and may be associated with increased duplicates in the sequencing data. In contrast, a peak DNA fragment size significantly greater than 325 bp may indicate too much gDNA in the fragmentation reaction and may be associated with decreased percent-on-target performance in sequencing results.

4 Measure the concentration of each library by integrating under the entire peak.

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

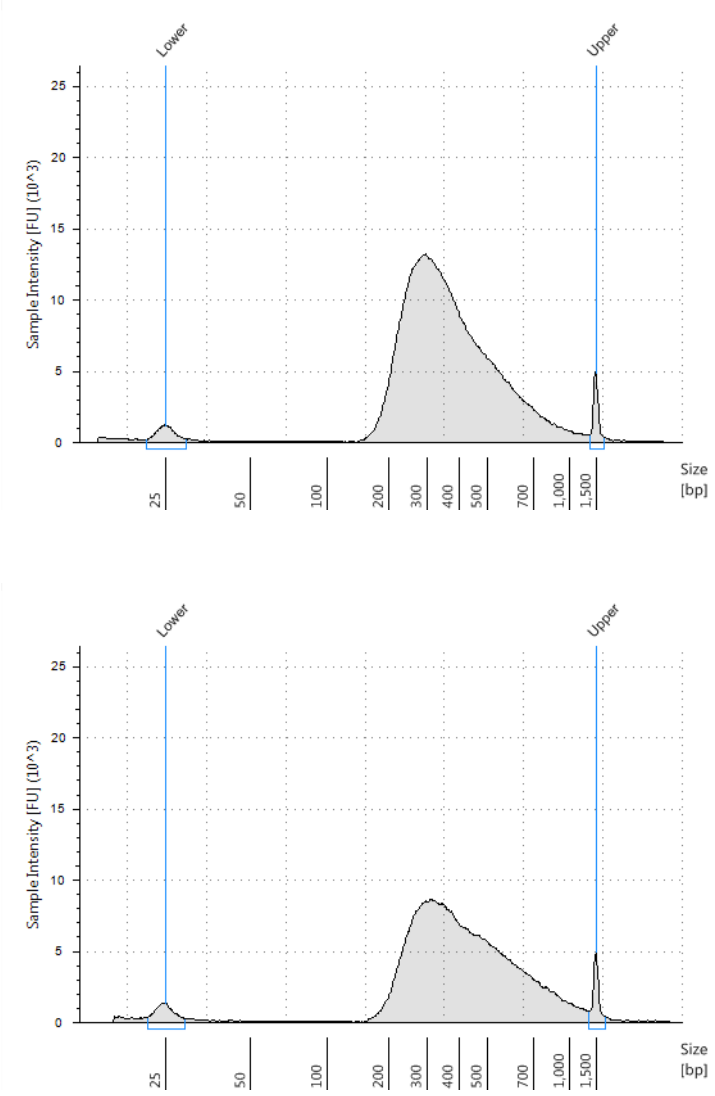


Figure 6 Representative sample electropherograms showing pre-capture analysis of amplified library DNA using the 2200 TapeStation with a D1000 ScreenTape.

4 Hybridization

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

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

Step 1. Aliquot prepped DNA samples for hybridization

For each sample library prepared, do one hybridization and capture. Do not pool samples at this stage.

The amount of prepared gDNA library used in the hybridization reaction varies according to the size of the Capture Library used for hybridization as outlined in Table 24 below. Use the maximum possible amount of each prepped DNA, within the range listed in Table 24.

Table 24 Amount of adaptor-tagged DNA libraries used for hybridization

Capture Library Size	Amount of prepared gDNA library used in hybridization
Libraries >3.0 Mb (except ClearSeq DNA Kinome; see below)	750 to 1500 ng DNA
Libraries ≤3.0 Mb and ClearSeq DNA Kinome (3.2 Mb)	500 to 750 ng DNA

Using the DNA concentration for each sample determined on page 58 to page 60, calculate the volume of each sample to be used for hybridization using the appropriate formula below:

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

OR

Volume (µl) = 1500 ng/concentration (ng/µl)

If the concentration of any sample is not sufficient to allow use of the recommended amount of DNA (750 ng for ClearSeq DNA Kinome and libraries ≤3.0 Mb or 1500 ng for libraries >3.0 Mb), then use the full remaining volume of DNA sample (approximately 12 µl) for the hybridization step.

The automation protocol 05 Aliquot_Libraries_v1.0.pro is used to prepare a new sample plate containing the appropriate amount of each DNA sample for hybridization. Before running the automation protocol, you must create a table containing instructions for the NGS Bravo indicating the volume of each sample to aliquot, as described in the steps below.

1 Create a .csv (comma separated value) file with the headers shown in Figure 7. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in .csv format. The file must include rows for all 96 wells of the plate.

2 Enter the information requested in the header for each DNA sample.

- In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
- In the SourceWell and DestinationWell fields, enter each well position for the plate. SourceWell and DestinationWell field contents must be identical for a given sample.
- In the Volume field, enter the volume (in µl) of each DNA sample to be used in the hybridization step (see page 64 for guidelines). For all empty wells on the plate, enter the value 0, as shown in Figure 7; **do not delete rows for empty wells**.

	A	B	C	D
1	**SourceBC**	**SourceWell**	**DestinationWell**	**Volume**
2	SamplePlateXYZ	A1	A1	5.35
3	SamplePlateXYZ	B1	B1	4.28
4	SamplePlateXYZ	C1	C1	4.76
5	SamplePlateXYZ	D1	D1	5.19
6	SamplePlateXYZ	E1	E1	5.49
7	SamplePlateXYZ	F1	F1	4.86
8	SamplePlateXYZ	G1	G1	5.05
9	SamplePlateXYZ	H1	H1	4.37
10	SamplePlateXYZ	A2	A2	0
11	SamplePlateXYZ	B2	B2	0
12	SamplePlateXYZ	C2	C2	0
13	SamplePlateXYZ	D2	D2	0

Figure 7 Sample spreadsheet for 1-column run.

NOTE

You can find a sample spreadsheet in the directory **C: > VWorks Workspace > NGS Option A> QXT_ILM_v1.0 > Aliquot Library Input Files > Aliquot_Libraries_full_plate_template.csv.**

The Aliquot_Libraries_full_plate_template.csv file may be copied and used as a template for creating the .csv files for each 05 Aliquot_Libraries_v1.0.pro run. If you are using the sample file as a template for runs with fewer than 12 columns, be sure to retain rows for all 96 wells, and populate the Volume column with 0 for unused wells.

3 Load the .csv file onto the PC containing the VWorks software into a suitable folder, such as **C: > VWorks Workspace > NGS Option A> QXT_ILM_v1.0 > Aliquot Library Input Files.**

Setup and Run VWorks protocol 05 Aliquot_Libraries_v1.0.pro

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 position 9 as indicated in Table 25. See page 21 for more information on how to do this step.

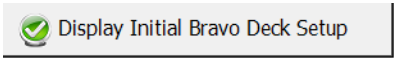
Table 25 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
9	0°C	ThermoCube control panel

3 On the SureSelect setup form, under **Select Protocol**, select **05 Aliquot_Libraries_v1.0.pro.**

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

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



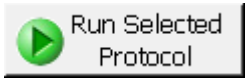
6 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 26.

Table 26 Initial Bravo deck configuration for 05 Aliquot_Libraries_v1.0.pro

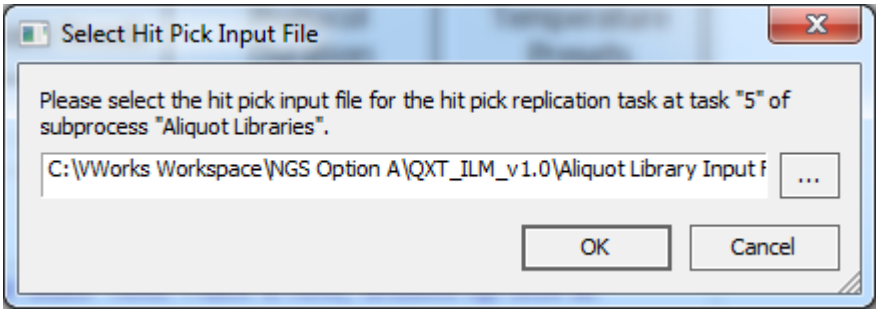
Location	Content
1	–(empty)–
2	–(empty)–
3	–(empty)–
4	–(empty)–
5	Empty Eppendorf plate
6	Empty tip box
7	–(empty)–
8	New tip box
9	Prepped library DNA in Eppendorf plate

CAUTION This protocol does not use the Current Tip State indicator function. Be sure to place a completely full box of tips at position 8 and a completely empty tip box at position 6.

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



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



The library aliquoting protocol takes approximately 1 hour for 96 samples. When complete, the DNA sample plate is on Bravo deck position 5.

9 Remove the sample plate from the Bravo deck and use a vacuum concentrator to dry the samples at ≤ 45°C.

10 Reconstitute each dried sample with 12 µl of nuclease-free water. Pipette up and down along the sides of each well for optimal recovery.

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

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

Step 2. Hybridize the gDNA library and SureSelect or ClearSeq Capture Library

In this step, automation protocol 06 Hybridization_QXT_v1.0.pro is used to complete the liquid handling steps to set up the hybridization reactions. Afterward, you transfer the sample plate to a thermal cycler, held at 65°C, to allow hybridization of the DNA samples to the Capture Library.

This step uses the SureSelect^{QXT} Reagent Kit components listed in Table 27. Thaw each component under the conditions indicated in the table. Vortex each reagent to mix, then spin briefly to collect the liquid.

Table 27 Reagents for Hybridization and Capture

Kit Component	Storage Location	Thawing Conditions	Where Used
SureSelect QXT Fast Hybridization Buffer	SureSelect QXT Hyb Module Box 2, –20°C	Warm to Room Temperature (RT), then keep at RT	page 75
SureSelect QXT Fast Blocker Mix	SureSelect QXT Hyb Module Box 2, –20°C	Thaw on ice	page 72
SureSelect RNase Block	SureSelect QXT Hyb Module Box 2, –20°C	Thaw on ice	page 73 or page 74
Capture Library	–80°C	Thaw on ice	page 73 or page 74

Program the thermal cycler

1 Pre-program the thermal cycler for the Hybridization workflow by entering the thermal cycling program shown in Table 28 below.

It is critical to pre-program the thermal cycler before starting the automation protocol for Hybridization, in order to maintain the required sample and reagent temperatures during the workflow.

Table 28 Thermal cycler program for Hybridization*

Segment Number	Purpose	Number of Cycles	Temperature	Time
1	Denaturation	1	95°C	5 minutes
2	Blocking	1	65°C	10 minutes
3	Hold for NGS Bravo steps†	1	65°C	Hold
4	Hybridization	60	65°C	1 minute
			37°C	3 seconds
5	Hold until start of Capture‡	1	65°C	Hold

* When setting up the thermal cycling program, use a reaction volume setting of 35 µl (final volume of hybridization reactions during cycling in Segment 4).

† Samples are transferred to the NGS Bravo during this Hold step when prompted by the VWorks software.

‡ Samples are held at 65°C until they are processed in the Capture & Wash automation protocol that begins on page 83.

CAUTION The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.

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 and 6 as indicated in Table 29. See page 19 for more information on how to do this step.

Table 29 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
4	23°C	Inheco Multi TEC control touchscreen (CPAC 2-1)
6	23°C	Inheco Multi TEC control touchscreen (CPAC 2-2)

3 Place a red PCR plate insert at Bravo deck position 4.

4 Place the silver Nunc DeepWell plate insert on position 6 of the Bravo deck. This insert is required to facilitate heat transfer to DeepWell source plate wells during the Hybridization protocol. When loading a source plate on the silver insert, make sure the plate is seated properly to ensure proper heat transfer.

Prepare the Block Master Mix

5 Prepare the appropriate volume of Block Master Mix, on ice, as indicated in Table 30.

Table 30 Preparation of Block 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	2.5 µl	31.9 µl	53.1 µl	74.4 µl	95.6 µl	138.1 µl	276.3 µl
SureSelect QXT Fast Blocker Mix (blue cap)	5.0 µl	63.8 µl	106.3 µl	148.8 µl	191.3 µl	276.3 µl	552.5 µl
Total Volume	**7.5 µl**	**95.6 µl**	**159.4 µl**	**223.1 µl**	**286.9 µl**	**414.4 µl**	**828.8 µl**

Prepare one or more Capture Library Master Mixes

6 Prepare the appropriate volume of Capture Library Master Mix for each library that will be used for hybridization as indicated in Table 31 to Table 34. Mix thoroughly by vortexing at high speed then spin down briefly. Keep the Capture Library Master Mix(es) on ice.

NOTE

Each row of the prepped gDNA sample plate may be hybridized to a different Capture Library. However, libraries of different sizes require different post-capture amplification cycles. Plan experiments such that similar-sized libraries are hybridized on the same plate.

For runs that use a single Capture Library for all rows of the plate, prepare the master mix as described in Step a (Table 31 or Table 32) on page 73.

For runs that use different Capture Libraries for individual rows, prepare each master mix as described in Step b (Table 33 or Table 34) on page 74.

a **For runs that use a single Capture Library for all rows**, prepare a Master Mix as described in Table 31 or Table 32, according to the size of the Capture Library.

Table 31 Preparation of Capture Library Master Mix for **Capture Libraries <3 Mb**, 8 rows of well

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µl	76.5 µl	114.8 µl	153.0 µl	191.3 µl	306.0 µl	592.9 µl
RNase Block (purple cap)	0.5 µl	8.5 µl	12.8 µl	17.0 µl	21.3 µl	34.0 µl	65.9 µl
Capture Library	2.0 µl	34.0 µl	51.0 µl	68.0 µl	85.0 µl	136.0 µl	263.5 µl
Total Volume	**7.0 µl**	**119.0 µl**	**178.6 µl**	**238.0 µl**	**297.6 µl**	**476.0 µl**	**922.3 µl**

Table 32 Preparation of Capture Library Master Mix for **Capture Libraries ≥3 Mb**[*], 8 rows of wells

Target size >3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µl	25.5 µl	38.3 µl	51.0 µl	63.8 µl	102.0 µl	197.6 µl
RNase Block (purple cap)	0.5 µl	8.5 µl	12.8 µl	17.0 µl	21.3 µl	34.0 µl	65.9 µl
Capture Library	5.0 µl	85.0 µl	127.5 µl	170.0 µl	212.5 µl	340.0 µl	658.8 µl
Total Volume	**7.0 µl**	**119.0 µl**	**178.6 µl**	**238.0 µl**	**297.6 µl**	**476.0 µl**	**922.3 µl**

* Includes ClearSeq DNA Kinome XT Library (3.2 Mb)

b **For runs that use different Capture Libraries in individual rows**, prepare a Master Mix for each Capture Library as listed in Table 33 or Table 34, according to the library size. The volumes listed in Table 33 and Table 34 are for a single row of sample wells. If a given Capture Library will be hybridized in multiple rows, multiply each of the values below by the number of rows assigned to that Capture Library.

Table 33 Preparation of Capture Library Master Mix for **Capture Libraries <3 Mb**, single row of wells

Target size <3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	4.5 µl	9.0 µl	13.8 µl	18.6 µl	23.3 µl	37.7 µl	73.5 µl
RNase Block (purple cap)	0.5 µl	1.0 µl	1.5 µl	2.1 µl	2.6 µl	4.2 µl	8.2 µl
Capture Library	2.0 µl	4.0 µl	6.1 µl	8.3 µl	10.4 µl	16.8 µl	32.7 µl
Total Volume	**7.0 µl**	**14.0 µl**	**21.4 µl**	**28.9 µl**	**36.3 µl**	**58.6 µl**	**114.4 µl**

Table 34 Preparation of Capture Library Master Mix for **Capture Libraries ≥3 Mb**[*], single row of wells

Target size >3.0 Mb							
Reagent	**Volume for 1 Library**	**Volume for 1 Column**	**Volume for 2 Columns**	**Volume for 3 Columns**	**Volume for 4 Columns**	**Volume for 6 Columns**	**Volume for 12 Columns**
Nuclease-free water	1.5 µl	3.0 µl	4.6 µl	6.2 µl	7.8 µl	12.6 µl	24.5 µl
RNase Block (purple cap)	0.5 µl	1.0 µl	1.5 µl	2.1 µl	2.6 µl	4.2 µl	8.2 µl
Capture Library	5.0 µl	10.0 µl	15.3 µl	20.6 µl	25.9 µl	41.9 µl	81.7 µl
Total Volume	**7.0 µl**	**14.0 µl**	**21.4 µl**	**28.9 µl**	**36.3 µl**	**58.6 µl**	**114.4 µl**

[*] Includes ClearSeq DNA Kinome XT Library (3.2 Mb)

Prepare the Hybridization Buffer master mix

7 Prepare the appropriate volume of Hybridization Buffer Master Mix, **at room temperature**, as indicated in Table 35.

Table 35 Preparation of Hybridization Buffer 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	2.5 µl	53.1 µl	74.4 µl	95.6 µl	116.9 µl	159.4 µl	297.5 µl
SureSelect QXT Fast Hybridization Buffer (yellow cap)	6.0 µl	127.5 µl	178.5 µl	229.5 µl	280.5 µl	382.5 µl	714.0 µl
Total Volume	**8.5 µl**	**180.6 µl**	**252.9 µl**	**325.1 µl**	**397.4 µl**	**541.9 µl**	**1011.5 µl**

Prepare the master mix source plate

8 In a Nunc DeepWell plate, prepare the master mix source plate containing the master mixes prepared in step 5 to step 7 at room temperature. Add the volumes indicated in Table 36 of each master mix to each well of the indicated column of the Nunc DeepWell plate. When using multiple Capture Libraries in a run, add each Capture Library Master Mix to the appropriate row(s) of the Nunc DeepWell plate. The final configuration of the master mix source plate is shown in Figure 8.

Table 36 Preparation of the Master Mix Source Plate for 06 Hybridization_QXT_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
Block Master Mix	Column 1 (A1-H1)	11.0 µl	19.0 µl	27.0 µl	34.9 µl	50.9 µl	102.7 µl
Capture Library Master Mix	Column 2 (A2-H2)	14.0 µl	21.4 µl	28.9 µl	36.3 µl	58.6 µl	114.4 µl
Hybridization Buffer Master Mix	Column 3 (A3-H3)	19.9 µl	29.0 µl	38.0 µl	47.0 µl	65.1 µl	123.8 µl

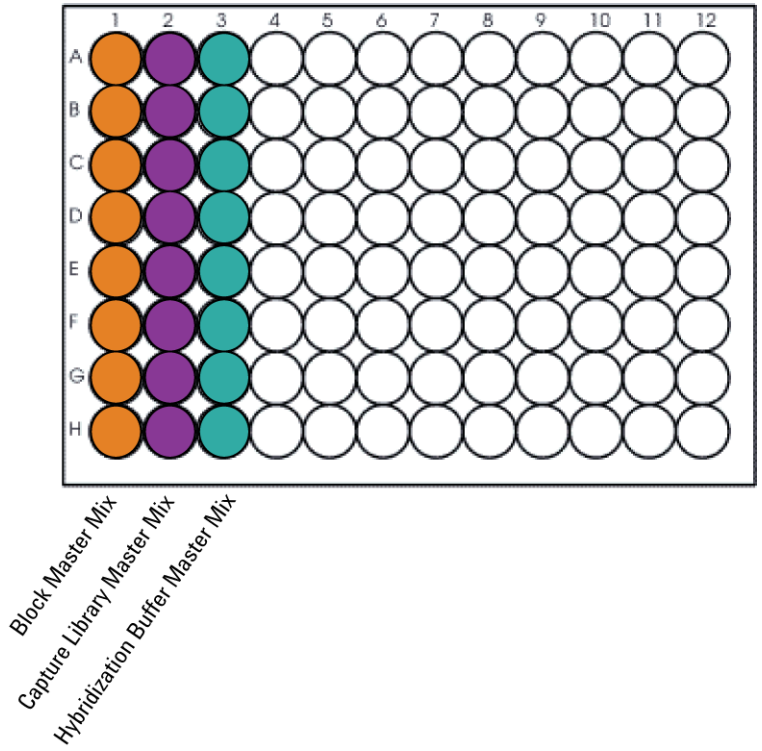


Figure 8 Configuration of the master mix source plate for 06 Hybridization_QXT_v1.0.pro.

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

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

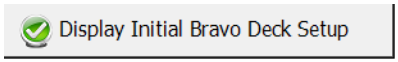
Setup and run VWorks protocol 06 Hybridization_QXT_v1.0.pro

11 On the SureSelect setup form, under **Select Protocol**, select **06 Hybridization_QXT_v1.0.pro.**

12 Under **Select PCR Plate Labware**, select the plate type to be used for the hybridization step (to be loaded at Bravo deck position 4).

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

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



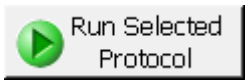
15 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 37.

Table 37 Initial Bravo deck configuration for 06 Hybridization_QXT_v1.0.pro

Location	Content
1	–(empty)–
2	New tip box
3	–(empty)–
4	Empty PCR plate, seated in red insert (PCR plate type must be specified on setup form under step 2)
5	Empty Eppendorf twin.tec plate
6	Master Mixes in Columns 1-3 of Nunc DeepWell, seated in silver Nunc DeepWell insert
7	–(empty)–
8	Empty tip box
9	Prepped library aliquots in Eppendorf twin.tec plate

16 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.

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



Running the 06 Hybridization_QXT_v1.0.pro protocol takes approximately 30 minutes. An operator must be present during the run to complete tip box replacement and other labware transfer steps, as directed by the VWorks prompts detailed below.

The NGS Bravo combines the prepped gDNA in the wells of the sample plate with the aliquotted SureSelect Block Master Mix. When this process is complete, you will be prompted to transfer the plate to the thermal cycler for sample denaturation and blocking prior to hybridization.

18 When prompted by VWorks as shown below, remove the PCR plate from position 4 of the Bravo deck, leaving the red insert in place. After removing the sample plate, click **Continue**.

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

20 Transfer the sealed plate to a thermal cycler and initiate the preprogrammed thermal cycling program described in Table 28 on page 70. The denaturation and blocking segments of the preprogrammed thermal cycler program are shown in Figure 9 below for reference.

Table 28 Thermal cycler program for Hybridization*

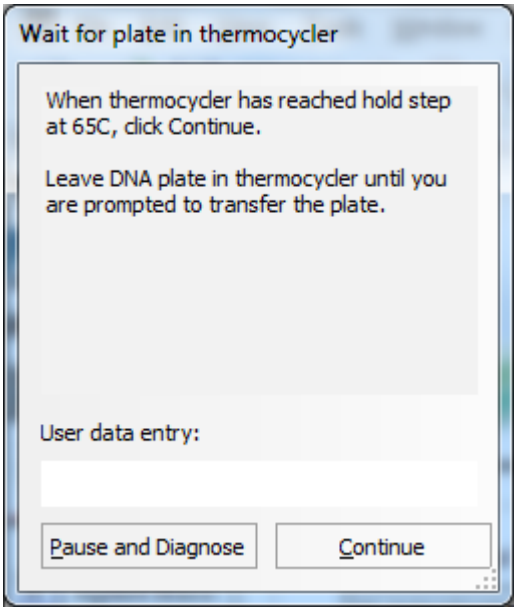
Segment Number	Purpose	Number of Cycles	Temperature	Time
1	Denaturation	1	95°C	5 minutes
2	Blocking	1	65°C	10 minutes
3	Hold for NGS workstation steps†	1	65°C	Hold

Figure 9 Preprogrammed thermal cycler segments used for sample denaturation and blocking prior to hybridization.

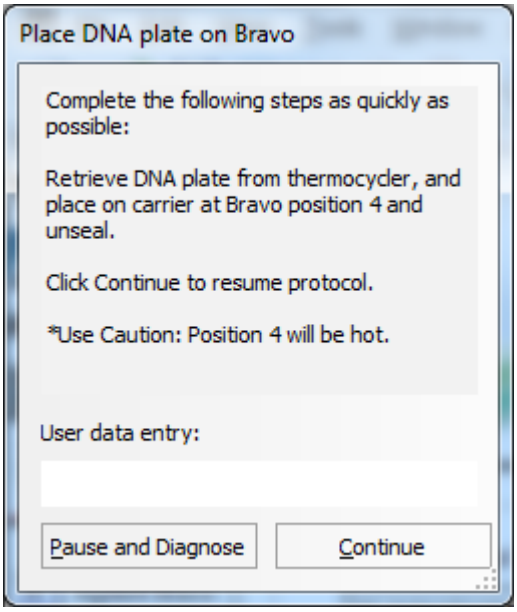
While the sample plate incubates on the thermal cycler, the NGS Bravo combines aliquots of the Capture Library Master Mix and Hybridization Buffer Master Mix.

CAUTION You must complete step 21 to step 25 quickly, and immediately after being prompted by the VWorks software. It is important that sample temperature remains approximately 65°C during transfers between the NGS Bravo and thermal cycler.

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



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



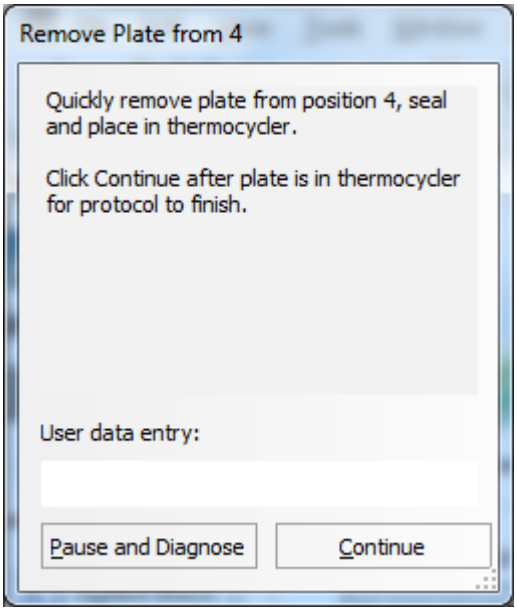
WARNING

Bravo deck position 4 will be hot.

Use caution when handling components that contact heated deck positions.

The NGS Bravo transfers the Capture Library-Hybridization Buffer mixture to the wells of the PCR plate that contain the mixture of prepped gDNA samples and blocking agents.

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



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

25 Quickly transfer the plate back to the thermal cycler, held at 65°C. Press the *Play* button to initiate the hybridization segment of the pre-programmed thermal cycling program (segment 4 from Table 28 on page 70). During this step, the prepared DNA samples are hybridized to the SureSelect or ClearSeq Capture Library.

CAUTION The thermal cycler is held at 65°C using a heated lid at 105°C. The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.

26 After initiating hybridization on the thermal cycler, click **Continue** on the VWorks screen to finish the NGS Bravo protocol.

During the thermal cycler incubation for hybridization (approximately 1.5-hour duration), complete the reagent and NGS Bravo setup steps for the capture automation protocol as described on page 83 to page 85.

Step 3. Capture the hybridized DNA

This step uses automation protocol 07 SureSelectQXT_Capture_v1.0.pro to automate capture of the gDNA-Capture Library hybrids using streptavidin-coated magnetic beads. Setup tasks for the Capture protocol (step 1, below, through step 16 on page 86) should be completed during the thermal cycler incubation for hybridization (approximately 1.5-hour duration) started on page 82.

This step uses the SureSelect^{QXT} Reagent Kit components in Table 38 in addition to streptavidin-coated magnetic beads obtained from another supplier (see Table 1 on page 12).

Table 38 Reagents for hybrid capture

Kit Component	Storage Location	Where Used
SureSelect Binding Buffer	SureSelect QXT Hyb Module Box 1, RT	page 84
SureSelect Wash Buffer 2	SureSelect QXT Hyb Module Box 1, RT	page 85

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 position 4 as indicated in Table 39. See page 19 for more information on how to do this step.

Table 39 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
4	66°C	Inheco Multi TEC control touchscreen (CPAC 2-1)

3 Place a red PCR plate insert at Bravo deck position 4.

4 Place the silver Nunc DeepWell plate insert at Bravo deck position 6. This insert is required to facilitate heat transfer to DeepWell source plate wells.

Prepare the Dynabeads streptavidin beads and Wash Buffer 2 source plates

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

6 Wash the magnetic beads.

a In a conical vial, combine the components listed in Table 40. The volumes below include the required overage.

Table 40 Magnetic bead washing mixture

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

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

c Put the vial into a magnetic device, such as the Dynal magnetic separator.

d Remove and discard the supernatant.

e Repeat step a through step d for a total of 3 washes. (Retain the beads after each wash and combine with a fresh aliquot of the indicated volume of SureSelect Binding Buffer.)

7 Resuspend the beads in SureSelect Binding buffer, according to Table 41 below.

Table 41 Preparation of magnetic beads for 07 SureSelectQXT_Capture_v1.0.pro

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
SureSelect Binding Buffer	0.2 ml	1.7 ml	3.3 ml	4.9 ml	6.6 ml	10 ml	20 ml

8 Prepare a Nunc DeepWell source plate for the washed streptavidin bead suspension. For each well to be processed, add 200 µl of the homogeneous bead suspension to the Nunc DeepWell plate.

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

10 Prepare a Nunc DeepWell source plate labeled *Wash #2*. For each well to be processed, add 1150 µl of SureSelect Wash Buffer 2. Place the Wash #2 source plate at position 6 of the Bravo deck.

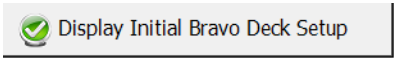
Setup VWorks protocol 07 SureSelectQXT_Capture_v1.0.pro

11 On the SureSelect setup form, under **Select Protocol**, select **07 SureSelectQXT_Capture_v1.0.pro.**

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

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

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



15 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 42.

Table 42 Initial Bravo deck configuration for 07 SureSelectQXT_Capture_v1.0.pro

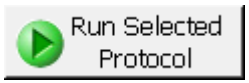
Location	Content
1	–(empty)–
2	New tip box
3	–(empty)–
4	**Empty** red insert
5	Prepared Dynabeads streptavidin bead DeepWell source plate
6	*Wash #2* DeepWell source plate seated on silver Nunc DeepWell insert
7	–(empty)–
8	Empty tip box
9	–(empty)–

16 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.

Run VWorks protocol 07 SureSelectQXT_Capture_v1.0.pro

Start the 07 SureSelectQXT_Capture_v1.0.pro protocol upon completion of the hybridization incubation that was started on page 82, when the thermal cycler program reaches the 65°C Hold step in Segment 5.

After verifying that the hybridization step is complete and that all NGS Bravo setup steps for capture are complete, click **Run Selected Protocol**. Leave the hybridization plate in the thermal cycler until you are prompted to transfer the plate to the NGS Bravo.

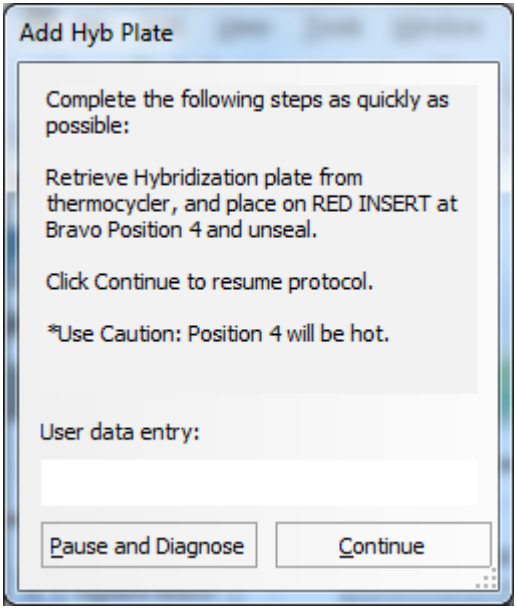


The total duration of the 07 SureSelectQXT_Capture_v1.0.pro protocol is approximately 35 minutes. An operator must be present to transfer the hybridization plate from the thermal cycler when prompted by VWorks as shown in step 17 below (<5 minutes after starting the protocol).

If the temperature of Bravo deck position 4 was not pre-set to 66°C, the protocol will pause while position 4 reaches temperature.

CAUTION It is important to complete step 17 quickly and carefully. Transfer the sample plate to the Bravo platform quickly to retain the 65°C sample temperature. Unseal the plate without tilting or jerking the plate to avoid sample splashing. Make sure that the NGS Bravo is completely prepared, with deck platforms at temperature and all components in place, before you transfer the sample plate to the Bravo deck.

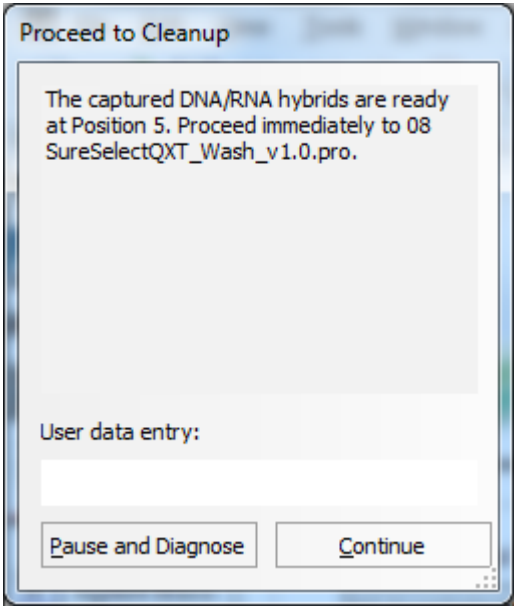
17 When prompted by VWorks as shown below, quickly remove the PCR plate, containing the hybridization reactions held at 65°C, from the thermal cycler. Unseal the plate carefully to avoid splashing, and quickly transfer the plate to position 4 of the Bravo deck, seated in the red insert. Click **Continue**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



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

Use caution when handling components that contact heated deck positions.

18 When the capture incubation period is complete you will be prompted by VWorks as shown below. Retain the hybrid-capture bead suspension plate at position 5 and proceed immediately to automation protocol 08 SureSelectQXT_Wash_v1.0.pro.



Step 4. Wash the captured DNA

This step uses automation protocol 08 SureSelectQXT_Wash_v1.0.pro to automate washing of the captured DNA-RNA hybrids.

This step uses the SureSelect^{QXT} Reagent Kit component in Table 38 in addition to components retained from the previous automation protocol.

Table 43 Reagents for capture wash protocol

Kit Component	Storage Location	Where Used
SureSelect Wash Buffer 1	SureSelect QXT Hyb Module Box 1, RT	page 89

Prepare the NGS Bravo

1 Retain all plates, inserts, and tip boxes used in the previous automation protocol on the Bravo deck, including the hybrid-capture bead suspension plate at position 5 and the pre-warmed Wash Buffer 2 source plate at position 6.

2 Pre-set the temperature of Bravo deck positions 4 and 6 as indicated in Table 29. See page 19 for more information on how to do this step.

Table 44 Bravo Deck Temperature Presets

Bravo Deck Position	Temperature Preset	Preset Method
4	69°C	Inheco Multi TEC control touchscreen (CPAC 2-1)
6	74°C	Inheco Multi TEC control touchscreen (CPAC 2-2)

3 Place an empty square-well plate waste reservoir at position 1 of the Bravo deck.

4 Prepare an Eppendorf source plate labeled *Wash #1.* For each well to be processed, add 160 µl of SureSelect Wash Buffer 1. Place the Wash #1 source plate at position 3 of the Bravo deck.

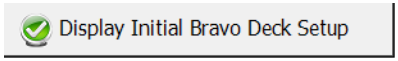
5 Prepare a Thermo Scientific reservoir containing 15 ml of nuclease-free water and place the reservoir at position 9 of the Bravo deck.

Setup and Run VWorks protocol 08 SureSelectQXT_Wash_v1.0.pro

6 On the SureSelect setup form, under **Select Protocol**, select **08 SureSelectQXT_Wash_v1.0.pro.**

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

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



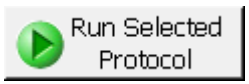
9 Verify that the Bravo deck has been set up according to the **Bravo Deck Setup** region of the form and as shown in Table 45.

Table 45 Initial Bravo deck configuration for 08 SureSelectQXT_Wash_v1.0.pro

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
2	New tip box
3	*Wash #*1 Eppendorf source plate
4	**Empty** red insert
5	DNA-RNA hybrids captured on streptavidin beads in DeepWell plate
6	*Wash #2* DeepWell source plate seated on silver Nunc DeepWell insert (pre-heated during Capture protocol)
7	–(empty)–
8	Empty tip box
9	Nuclease-free water reservoir

10 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.

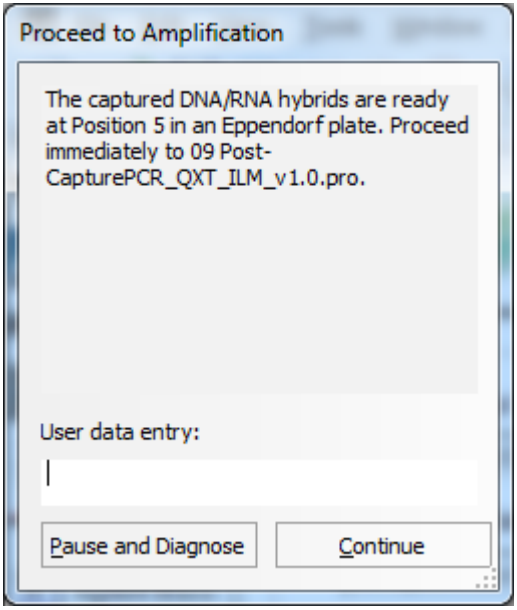
11 When setup and verification is complete, click **Run Selected Protocol**.



Running the 08 SureSelectQXT_Wash_v1.0.pro protocol takes approximately 60 minutes. An operator must be present during the run to complete tip box replacement as directed by VWorks prompts. Once complete, you will be prompted as shown below.

12 When the wash protocol is complete, the captured, bead-bound DNA samples are located in the Eppendorf plate at position 5 of the Bravo deck, and you will be prompted by VWorks as shown below. Click **Continue** on the VWorks screen to finish the protocol.

Remove the DNA sample plate from position 5 and seal the wells using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec. Store the plate on ice until it is used on page 101. Proceed immediately to the 09 Post-CapturePCR_QXT_ILM_v1.0.pro protocol, starting on page 94.



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

5 Indexing

This chapter describes the steps to add index tags by amplification, purify, assess quality and quantity of the libraries, and pool indexed samples for multiplexed sequencing.

Step 1. Amplify the captured DNA libraries to add index tags

In this step, the Agilent NGS Bravo completes the liquid handling steps for PCR-based addition of dual indexing tags to the captured DNA samples using automation protocol 09 Post-CapturePCR_QXT_ILM_v1.0.pro. After the PCR plate is prepared by the NGS Bravo, you transfer the plate to a thermal cycler for amplification.

This step uses the components listed in Table 46. Thaw then vortex to mix the reagents listed below and keep on ice.

Table 46 Reagents for post-capture indexing by PCR amplification

Kit Component	Storage Location	Where Used
Herculase II Fusion DNA Polymerase	SureSelect QXT Library Prep Kit Box 2, –20°C	page 95
Herculase II 5× Reaction Buffer	SureSelect QXT Library Prep Kit Box 2, –20°C	page 95
100 mM dNTP Mix (25 mM each dNTP)	SureSelect QXT Library Prep Kit Box 2, –20°C	page 95
SureSelect QXT P7 and P5 dual indexing primers	SureSelect QXT Library Prep Kit Box 2, –20°C	page 96

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 47. See page 19 to page 21 for more information on how to do this step.

Table 47 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

3 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

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

4 Prepare the appropriate volume of PCR master mix, according to Table 48. Mix well using a vortex mixer and keep on ice.

Table 48 Preparation of PCR Master Mix for 09 Post-CapturePCR_QXT_ILM_v1.0.pro,

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	9.5 µl	121.1 µl	201.9 µl	282.6 µl	363.4 µl	524.9 µl	1049.8 µl
Herculase II 5× Reaction Buffer	10.0 µL	127.5 µl	212.5 µl	297.5 µl	382.5 µl	552.5 µl	1105.0 µl
100 mM 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	**21.0 µl**	**267.8 µl**	**446.3 µl**	**624.8 µl**	**803.3 µl**	**1160.3 µl**	**2320.6 µl**

5 Using the same Nunc DeepWell master mix source plate that was used for the 06 Hybridization_QXT_v1.0.pro protocol, add the volume of PCR master mix indicated in Table 49 to all wells of column 4 of the plate. Keep the source plate on ice until it is used on page 97.

Table 49 Preparation of the Master Mix Source Plate for 09 Post-CapturePCR_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 4 (A4-H4)	30.8 µl	53.2 µl	75.5 µl	97.8 µl	142.4 µl	287.4 µl

NOTE If you are using a new DeepWell plate for the post-capture PCR source plate, leave columns 1 to 3 empty and add the PCR Master Mix to column 4 of the new plate.

Assign and aliquot 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 69 and Table 70 for sequencing on HiSeq and MiSeq platforms or see Table 69 and Table 71 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 target-enriched 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 details of P5 primer addition to the master mix source plate.)
- 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 target-enriched 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 72 on page 125 for additional details.

7 Dilute each P7 indexing primer (P7 i1 through P7 i12) to be used in the run according to Table 50. The volumes below include the required excess.

Table 50 Preparation of P7 indexing primer dilutions

Reagent	Volume to Index 1 Sample	Volume to Index 8 Samples
Nuclease-free water	4.0 µl	34 µl
SureSelect QXT P7 dual indexing primer (P7 i1 to P7 i12)	1.0 µl	8.5 µl
Total Volume	**5.0 µl**	**42.5 µl**

8 In a fresh PCR plate, aliquot 5 µl of the appropriate P7 indexing primer dilution from Table 50 to the intended sample indexing well position(s).

Keep the plate on ice.

9 Obtain the Nunc DeepWell master mix source plate containing the PCR Master Mix in column 4 (prepared in step 5, above). Add each P5 indexing primer (P5 i13 through P5 i20) to be used in the run to the master mix in the appropriate well of column 4. Add the volume listed in Table 51 to each well of column 4, according to the number of sample columns in the run. Each well of column 4 can contain the same or different P5 indexing primers. The final configuration of the master mix source plate is shown in Figure 10 on page 98.

Keep the source plate on ice.

Table 51 Addition of P5 indexing primers to the post-capture PCR master mix 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 4 (A4-H4)	1.5 µl	2.5 µl	3.6 µl	4.7 µl	6.8 µl	13.7 µl

* Each well of column 4 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.

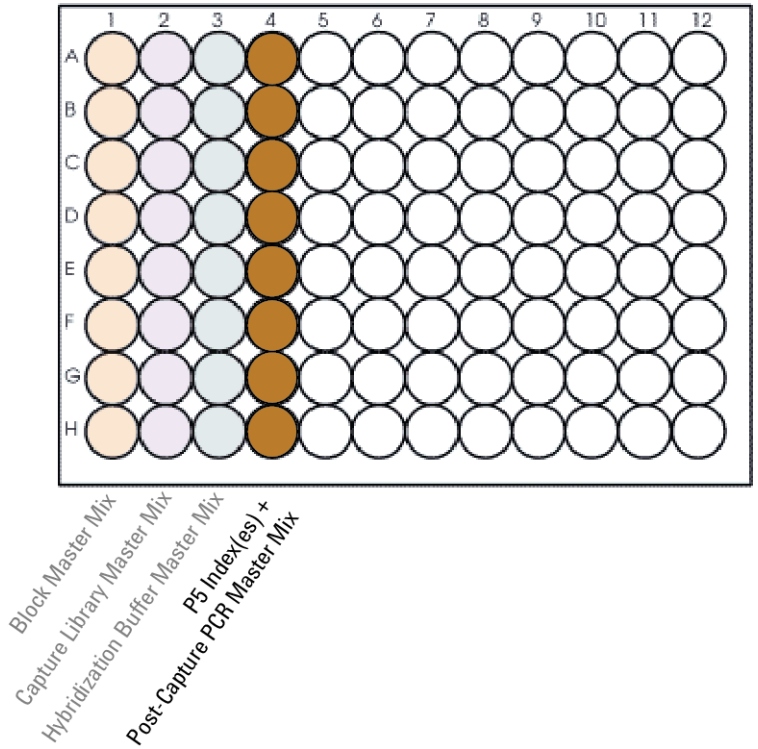


Figure 10 Configuration of the master mix source plate for 09 Post-CapturePCR_QXT_ILM_v1.0.pro.

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

11 Vortex the plate to ensure complete mixing, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

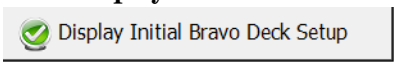
Setup and run VWorks protocol 09 Post-CapturePCR_QXT_ILM_v1.0.pro,

12 On the SureSelect setup form, under **Select Protocol to Run**, select **09 Post-CapturePCR_QXT_ILM_v1.0.pro.**

13 Under **Select PCR plate Labware**, select the specific type of PCR plate used at position 6 of the Bravo deck.

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

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



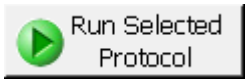
16 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 52.

Table 52 Initial Bravo deck configuration for 01 Tn_QXT_ILM_v1.0.pro

Location	Content
1	–(empty)–
2	New tip box
3	–(empty)–
4	–(empty)–
5	Captured DNA bead suspensions in Eppendorf twin.tec plate
6	Diluted P7 indexing primers in 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	Master mix plate containing P5 indexing primers and PCR Master Mix in Column 4 (unsealed), seated in silver insert

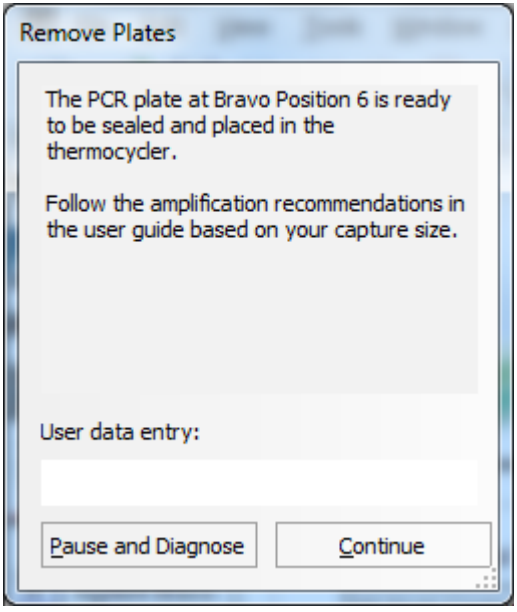
17 Verify that the Current Tip State indicator on the form matches the configuration of unused and used tips in the tip boxes at Bravo Deck positions 2 and 8, respectively. See page 23 for more information on using this segment of the form during the run.

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



19 Running the 09 Post-CapturePCR_QXT_ILM_v1.0.pro, protocol takes approximately 15 minutes. Once complete, the PCR-ready samples, containing captured DNA, PCR master mix, and indexing primers 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 53.

Table 53 Post-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	Capture Libraries >3 Mb: 10 Cycles	98°C	30 seconds
	Capture Libraries 1 to 3 Mb: 12 Cycles	58°C	30 seconds
	Capture Libraries <1 Mb: 14 Cycles	72°C	1 minute
3	1	72°C	5 minutes
4	1	4°C	Hold

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

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

This step uses protocol **10 Cleanup_Post-CapturePCR_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 54. See page 19 for more information on how to do this step.

Table 54 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 60 µl of homogeneous AMPure XP beads per well, for each well to be processed.

6 Prepare a Thermo Scientific Matrix reservoir containing 20 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.

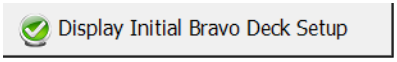
Setup and run VWorks 10 Cleanup_Post-CapturePCR_QXT_ILM_v1.0.pro

9 On the SureSelect setup form, under **Select Protocol**, select **10 Cleanup_Post-CapturePCR_QXT_ILM_v1.0.pro.**

10 Under **Select PCR plate Labware**, select the specific type of PCR plate used for post-capture amplification.

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**.



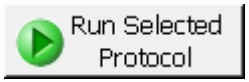
13 Load the Bravo deck according to the **Bravo Deck Setup** region of the form and as shown in Table 55.

Table 55 Initial Bravo deck setup for 10 Cleanup_Post-CapturePCR_QXT_ILM_v1.0.pro

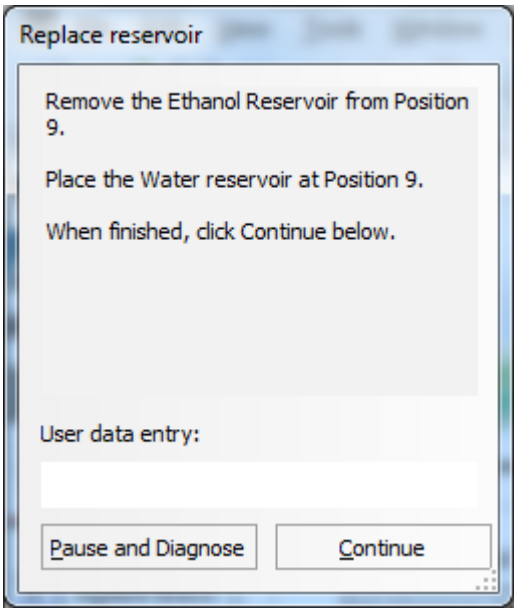
Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
2	New tip box
3	Empty Eppendorf plate
4	–(empty)–
5	AMPure XP beads in Nunc DeepWell plate (60 µl beads per processing well)
6	Amplified DNA libraries in unsealed PCR plate seated in red insert (PCR plate type must be 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 10 Cleanup_Post-CapturePCR_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.

Step 3. Assess indexed DNA quality

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

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

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

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

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

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

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

5 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.

6 Verify that the electropherogram shows the peak of DNA fragment size positioned between 325 and 450 bp. A sample electropherogram is shown in Figure 11.

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.

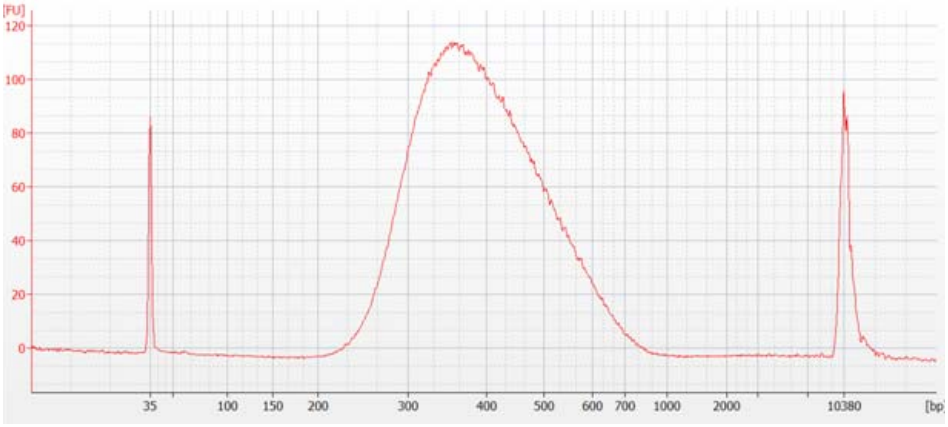


Figure 11 Analysis of indexed DNA using the High Sensitivity DNA Assay.

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

Use a High Sensitivity D1000 ScreenTape (p/n 5067-5584) and reagent kit (p/n 5067-5585) to analyze the indexed DNA using the Agilent 4200 TapeStation or 2200 TapeStation. For more information to do this step, see the appropriate TapeStation user manual at www.genomics.agilent.com.

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

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

3 Prepare the TapeStation samples as instructed in the instrument user manual. Use 2 µl of each indexed DNA sample diluted with 2 µl of High Sensitivity D1000 sample buffer for the analysis.

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

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

5 Verify that the electropherogram shows the peak of DNA fragment size positioned between 325 and 450 bp. A sample electropherogram is shown in Figure 12.

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

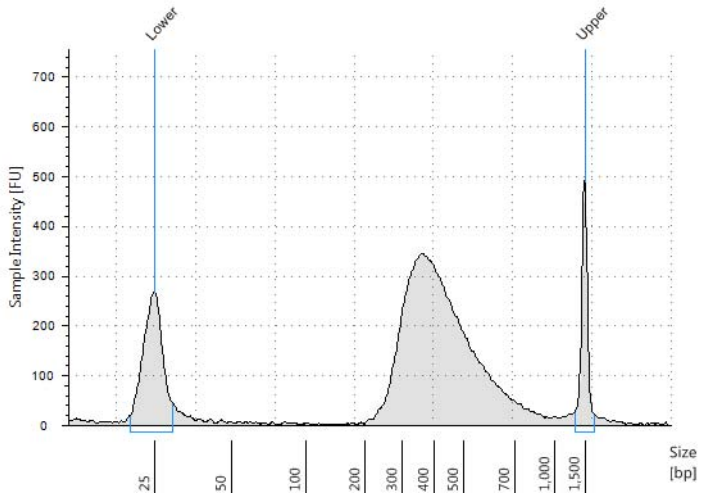


Figure 12 Analysis of indexed DNA using the 2200 TapeStation.

Step 4. Quantify each index-tagged library by QPCR (optional)

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

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

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

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

Step 5. Pool samples for Multiplexed Sequencing

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

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

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

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

is the number of indexes, and

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

Table 56 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 56 Example of index volume calculation for a 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 6. Prepare sequencing samples

The optimal seeding concentration for SureSelect^{QXT} target-enriched libraries is 8 to 12 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 57 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 112.

Table 57 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 x 50 Cycle Kit†	v3
HiSeq 2500	High Output	2 × 100 bp	250 Cycle Kit	v4
HiSeq 2000	All Runs	2 × 100 bp	4 x 50 Cycle Kit†	v3
MiSeq	All Runs	2 × 100 bp	300 Cycle Kit	v2
MiSeq	All Runs	2 × 76 bp	150 Cycle Kit	v3
NextSeq 500	All Runs	2 × 100 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'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 Read Primer
- SureSelect QXT Index 2 Read Primer NSQ (NextSeq platform only)

These SureSelect^{QXT} custom sequencing primers are provided at 100 µM 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 58 or Table 59 on page 113.

For the MiSeq platform, combine the primers as shown in Table 60 on page 113.

For the NextSeq platform, combine the primers as shown in Table 61 or Table 62 on page 114.

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.

Table 58 **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 59 **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 60 **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 61 **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 62 **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

Step 7. 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 starting on page 123.

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.

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 13 on page 116. 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 116.

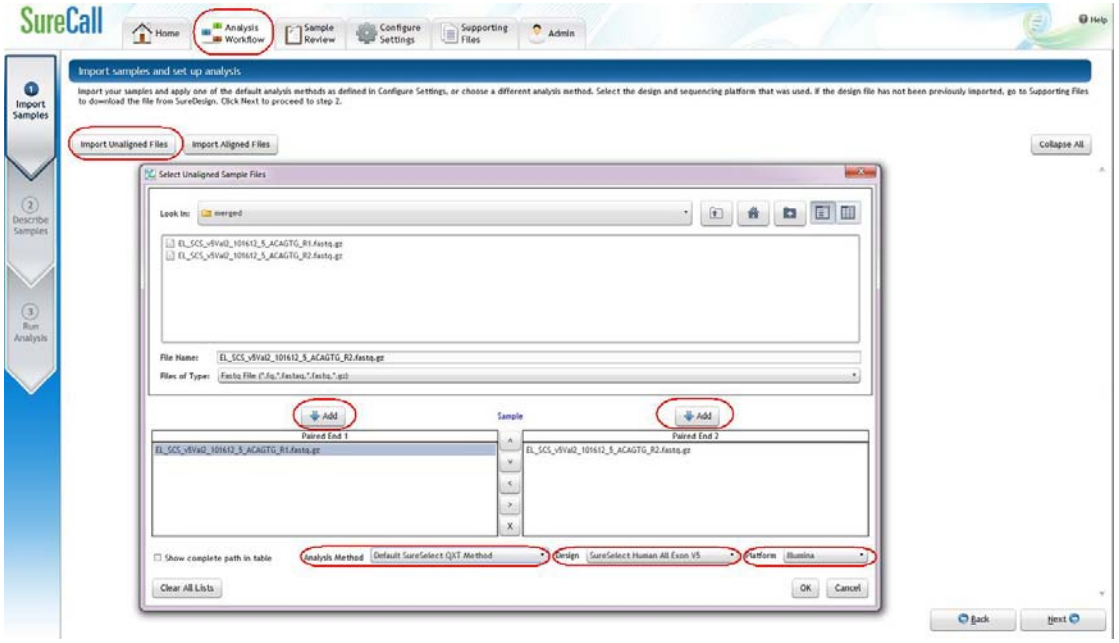


Figure 13 Analysis workflow setup in SureCall.

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 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 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.

Use the *Cycles* settings shown in Table 63. 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 63 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.

6 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} Reagent Kits contain the following component kits:

Table 64 SureSelect^{QXT} Reagent Kit Contents

Component Kits	Storage Condition	HiSeq or MiSeq (ILM) 96 Samples	NextSeq (NSQ) 96 Samples
SureSelect QXT Library Prep Kit, ILM, Box 2*	–20°C	5500-0121	5500-0127
SureSelect QXT Target Enrichment Kit, ILM Hyb Module, Box #1	Room Temperature	5190-7335	5190-7335
SureSelect QXT Target Enrichment Kit, ILM Hyb Module, Box #2	–20°C	5190-7334	5190-7334

* SureSelect QXT Library Prep Kit, ILM, Box 1 is not required for the workflow described in this manual.

The contents of each of the component kits listed in Table 64 are described in Table 66 to Table 68 below.

Table 65 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 66 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)

Table 67 SureSelect QXT Hyb Module Box 1 Content

Kit Component	96 Reactions
SureSelect QXT Stop Solution	bottle
SureSelect Binding Buffer	bottle
SureSelect Wash Buffer 1	bottle
SureSelect Wash Buffer 2	bottle

Table 68 SureSelect QXT Hyb Module Box 2 Content

Kit Component	96 Reactions
SureSelect QXT Fast Hybridization Buffer	bottle
SureSelect QXT Fast Blocker Mix	tube with blue cap
SureSelect QXT Primer Mix	tube with clear cap
SureSelect RNase Block	tube with purple cap

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 69 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 70 SureSelect^{QXT} 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 71 SureSelect^{QXT} 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 70.

Guidelines for Multiplexing with Dual-Indexed Samples

Agilent recommends following the dual index sample pooling guidelines and shown in Table 72. 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 72 Dual index sample pooling guidelines for 96 Reaction Kits

Plexity of Sample Pool	Recommended SureSelect^{QXT} P7 Indexes	Recommended SureSelect^{QXT} 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 Library Prep and Target Enrichment protocol using Agilent's NGS Bravo Option A.

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Agilent Technologies