SureSelect Automated Strand-Specific RNA Library Prep

Automated Poly-A Selection and Strand-Specific mRNA Library Preparation for the Illumina Platform

Protocol

Version F0, June 2020

SureSelect platform manufactured with Agilent SurePrint Technology

Agilent Technologies

Notices

Manual Part Number

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Acknowledgment

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In this Guide...

This guide describes an optimized protocol for Illumina paired-end multiplexed mRNA library preparation using the Agilent SureSelect Automated Strand-Specific Library Prep system.

This protocol is specifically developed and optimized to prepare mRNA sequencing libraries from total RNA samples. Sample processing steps are automated using the NGS Workstation.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Using the Agilent NGS Workstation for SureSelect RNA Library Preparation

This chapter contains an orientation to the Agilent NGS Workstation, an overview of the SureSelect mRNA Library Prep protocol, and considerations for designing SureSelect experiments for automated processing using the Agilent NGS Workstation.

3 Sample Preparation

This chapter describes the steps to prepare strand-specific libraries from total RNA samples for mRNA sequencing on the Illumina platform.

4 Reference

This chapter contains reference information.

What's New in Version F0

- Support for renamed Library Preparation Kit components. See Table 49 on page 88 and Table 50 on page 89 for a summary of component name changes. The new component names are utilized throughout the protocols in this document. No changes were made to the reagent formulations or methods of use.
- Support for revised Poly-A Selection Module subkit. See Table 49 on page 88 and Table 51 on page 90 for a summary of component name and configuration changes. The new component names are utilized throughout the protocols in this document. No changes were made to the reagent formulations or methods of use. Water is no longer provided in this module (see Table 51 on page 90) and has been added to the list of required reagents in Table 1 on page 12.
- Support for VWorks software version 13.1.0.1366 and Agilent NGS Workstation Option B p/n G5574AA (see Table 2 on page 13)
- Updates to Agilent 2100 Bioanalyzer system ordering information (see page 14)
- Support for Agilent 4150 TapeStation and addition of Agilent 4200/4150 TapeStation system-compatible plasticware ordering information (see page 14)
- Updates to mixing instructions for sample qualification assays using an Agilent TapeStation instrument (see *Caution* on page 81)
- Updates to ordering information for AMPure XP Kits (see Table 1 on page 12).
- Updated recommendation for control reference RNA (see Table 3 on page 14).
- Updates to sequencing support guidelines for RNA strandedness (see page 86) and recommended cycle number settings (see page 85).
- Updated *Notice to Purchaser* (see page 2).
- Updates to Technical Support contact information (see page 2)

Content

1

Before You Begin

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

NOTE

This protocol describes automated sample processing using the Agilent NGS Workstation. For non-automated sample processing procedures for Agilent's SureSelect Strand-Specific RNA Library Prep Kit for mRNA Sequencing on the Illumina platform, see publication G9691-90010.

Procedural Notes

- Certain protocol steps require the rapid transfer of sample plates between the Bravo deck and a thermal cycler. Locate your thermal cycler in close proximity to the Agilent NGS Workstation to allow rapid and efficient plate transfer.
- Use of Agilent's SureCycler 8800 thermal cycler and associated plasticware is recommended for optimal performance. The workflow is compatible with additional thermal cyclers, but performance should be validated before running a large number of samples. See page 30 for a list of supported PCR plate types and ensure that the thermal cycler to be used is compatible with one of the supported PCR plate types.
- Prepare and load the Agilent NGS Workstation as detailed in each of the protocol steps before initiating each automated protocol run. When loading plates in the workstation's Labware MiniHub, always place plates in the orientation shown in Figure 4 on page 37.
- 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.
- Avoid repeated freeze-thaw cycles of stock and diluted RNA and cDNA solutions. 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.
- When preparing master mix reagent stock solutions for use:
    1. Thaw the reagent vial as rapidly as possible without heating above room temperature.
    2. Mix thoroughly on a vortex mixer at high speed for 5 seconds, then briefly spin in a centrifuge to drive the contents off of walls and lid.
    3. Store vials used during an experiment on ice or in a cold block.
    4. Library Preparation Master Mixes should not be frozen and thawed more than five times. If you plan to use the reagents in more than five experiments, aliquot to multiple vials to minimize freeze/thaw cycles for each vial.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION

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

Required Reagents

Table 1 Required Reagents for SureSelect RNA Library Prep Automation

Description	Vendor and part number
SureSelect Strand Specific RNA Reagent Kit Illumina platforms (ILM), 96 Samples[*]	Agilent p/n G9691B
Actinomycin D[†]	Sigma p/n A1410
DMSO	Sigma p/n D8418
AMPure XP Kit 5 mL 60 mL 450 mL	Beckman Coulter Genomics p/n A63880 p/n A63881 p/n A63882
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific p/n AM9930

[*] Each 96-reaction kit contains sufficient reagents for 96 reactions used in runs that include at least 3 columns of samples per run.

[†] Actinomycin D should be obtained as a solid and prepared at 4 µg/µl concentration in DMSO then stored in single-use aliquots at –20°C, protected from light. The aliquots may be stored for up to one year before use. See page 34 for additional information.

Required Equipment

Table 2 Required Equipment for SureSelect RNA Library Prep Automation

Description	Vendor and part number
Agilent NGS Workstation Option B Contact Agilent Automation Solutions for more information: Customerservice.automation@agilent.com	Agilent p/n G5522A (VWorks software version 13.1.0.1366, 13.0.0.1360, or 11.3.0.1195) OR Agilent p/n G5574AA (VWorks software version 13.1.0.1366)
Bravo 96-well PCR plate insert (red)	Agilent p/n G5498B#13
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Thermal cycler and accessories	SureCycler 8800 Thermal Cycler (Agilent p/n G8810A), 96 well plate module (Agilent p/n G8810A) and compression mats (Agilent p/n 410187) or equivalent
PCR plates compatible with selected thermal cycler, e.g. Agilent semi-skirted PCR plate for the SureCycler 8800 Thermal Cycler When selecting plates for another thermal cycler, see page 30 for the list of PCR plates supported in automation protocols	Agilent p/n 401334
Eppendorf twin.tec full-skirted 96-well PCR plates	Eppendorf p/n 951020401 or 951020619
Thermo Scientific Reservoirs	Thermo Scientific p/n 1064156
Nunc DeepWell Plates, sterile, 1.3-mL well volume	Thermo Scientific p/n 260251
Axygen 96 Deep Well Plate, 2 mL, Square Well (waste reservoirs; working volume 2.2 mL)	Axygen p/n P-2ML-SQ-C E & K Scientific p/n EK-2440
Nucleic acid surface decontamination wipes	DNA Away Surface Decontaminant Wipes, Thermo Scientific p/n 7008, or equivalent
Vacuum concentrator	Savant SpeedVac, model DNA120, with 96-well plate rotor, model RD2MP, or equivalent
Low-Adhesion Tubes (RNase, DNase, and DNA-free) 1.5 mL 0.5 mL	USA Scientific p/n 1415-2600 p/n 1405-2600

Table 2 Required Equipment (continued)for SureSelect RNA Library Prep Automation

Description	Vendor and part number
DNA Analysis Platform and Consumables	
Agilent 2100 Bioanalyzer Instrument	Agilent p/n G2939BA
Agilent 2100 Expert SW Laptop Bundle (optional)	Agilent p/n G2953CA
DNA 1000 Kit	Agilent p/n 5067-1504
OR	
Agilent 4200 TapeStation*	Agilent p/n G2991AA
96-well sample plates	Agilent p/n 5042-8502
96-well plate foil seals	Agilent p/n 5067-5154
8-well tube strips	Agilent p/n 401428
8-well tube strip caps	Agilent p/n 401425
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
Nuclease-free 0.2 mL PCR tubes, thin-walled	Eppendorf p/n 951010006 or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Ice bucket	
Powder-free gloves	
Vortex mixer	
Timer	

* DNA samples may also be analyzed using the 4150 TapeStation, p/n G2992AA. ScreenTape devices and associated reagents listed in this table are compatible with both platforms.

Optional Reagents and Equipment

Table 3 Optional Reagents and Equipment

Description	Vendor and part number
Labnet MPS1000 Mini Plate Spinner	Labnet International p/n C1000
Agilent QPCR Human Reference Total RNA	Agilent p/n 750500

2

Using the Agilent NGS Workstation for SureSelect RNA Library Preparation

This chapter contains an orientation to the Agilent NGS Workstation, an overview of the SureSelect single-stranded RNA library preparation protocol, and considerations for designing SureSelect RNA experiments for automated processing using the Agilent NGS Workstation.

About the Agilent NGS Workstation

CAUTION Before you begin, make sure that you have read and understand operating, maintenance and safety instructions for using the Bravo platform and additional devices included with the workstation. Refer to the user guides listed in Table 4.

Review the user guides listed in Table 4 (available at Agilent.com) to become familiar with the general features and operation of the Agilent NGS Workstation Option B components. Instructions for using the Bravo platform and other workstation components for the SureSelect RNA Library Preparation workflow are detailed in this user guide.

Table 4 Agilent NGS Workstation components User Guide reference information

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

About the Bravo Platform

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

Bravo Platform Deck

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

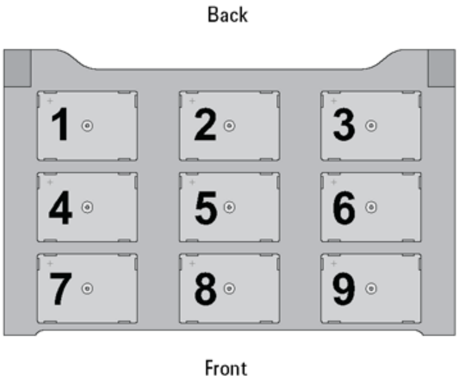


Figure 1 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

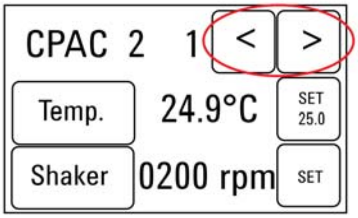
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the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

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

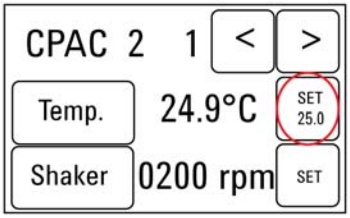
Table 5 Inheco Multi TEC Control touchscreen designations

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

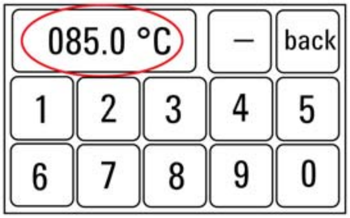
1. Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



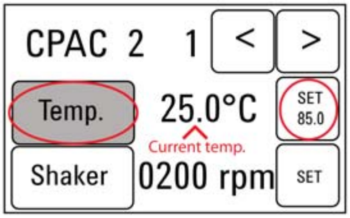
2. To set the temperature of the selected block, press the SET button.



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 will then initates temperature control of Bravo deck position 9 at the displayed set point.

VWorks Automation Control Software

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

NOTE

The instructions in this manual are compatible with VWorks software version 13.1.0.1366, 13.0.0.1360 or 11.3.0.1195.

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

Logging in to the VWorks software

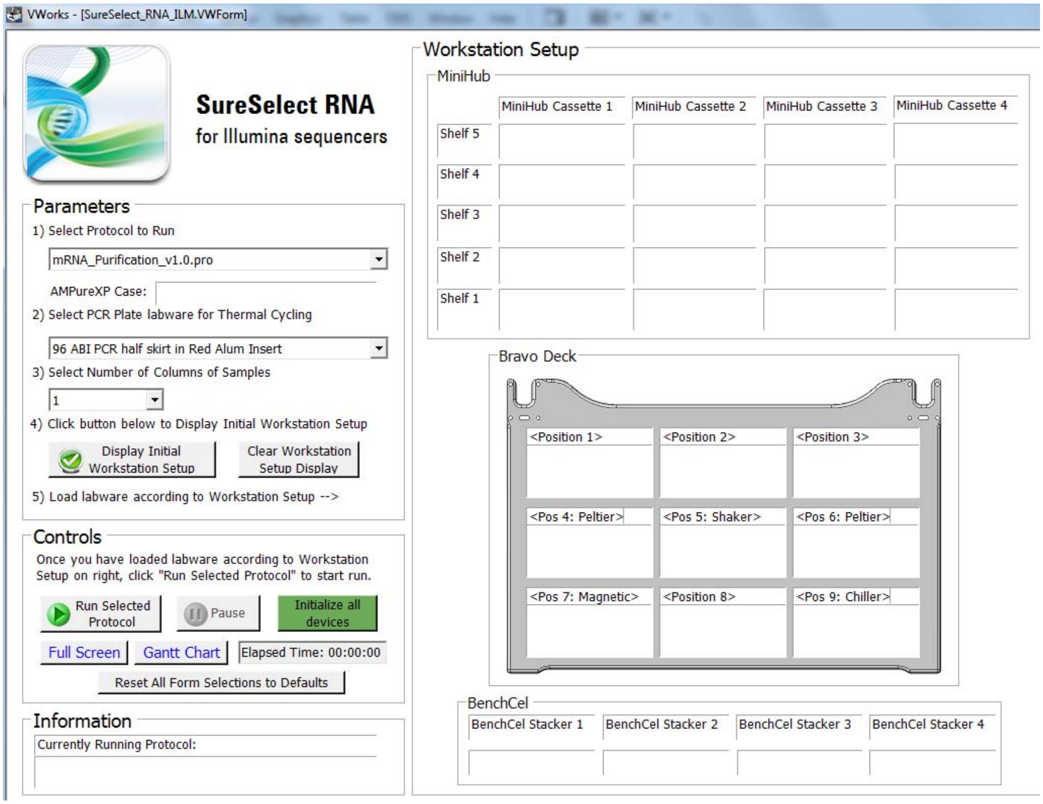
1. Double-click the VWorks icon or the SureSelect_RNA_ILM.VWForm shortcut on the Windows desktop to start the VWorks software.
2. If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
3. In the User Authentication dialog, type your VWorks user name and password,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

VWorks protocol and runset files

VWorks software uses two file types for automation runs, .pro (protocol) files and .rst (runset) files. Runset files are used for automated procedures in which the workstation uses more than one automation protocol during the run.

Using the SureSelect_RNA_ILM.VWForm to setup and start a run

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



VWorks - [SureSelect_RNA_ILM.VWForm]

SureSelect RNA
for Illumina sequencers

Parameters

1) Select Protocol to Run: mRNA_Purification_v1.0.pro

AMPureXP Case:

2) Select PCR Plate labware for Thermal Cycling: 96 ABI PCR half skirt in Red Alum Insert

3) Select Number of Columns of Samples: 1

4) Click button below to Display Initial Workstation Setup

5) Load labware according to Workstation Setup -->

Controls

Once you have loaded labware according to Workstation Setup on right, click "Run Selected Protocol" to start run.

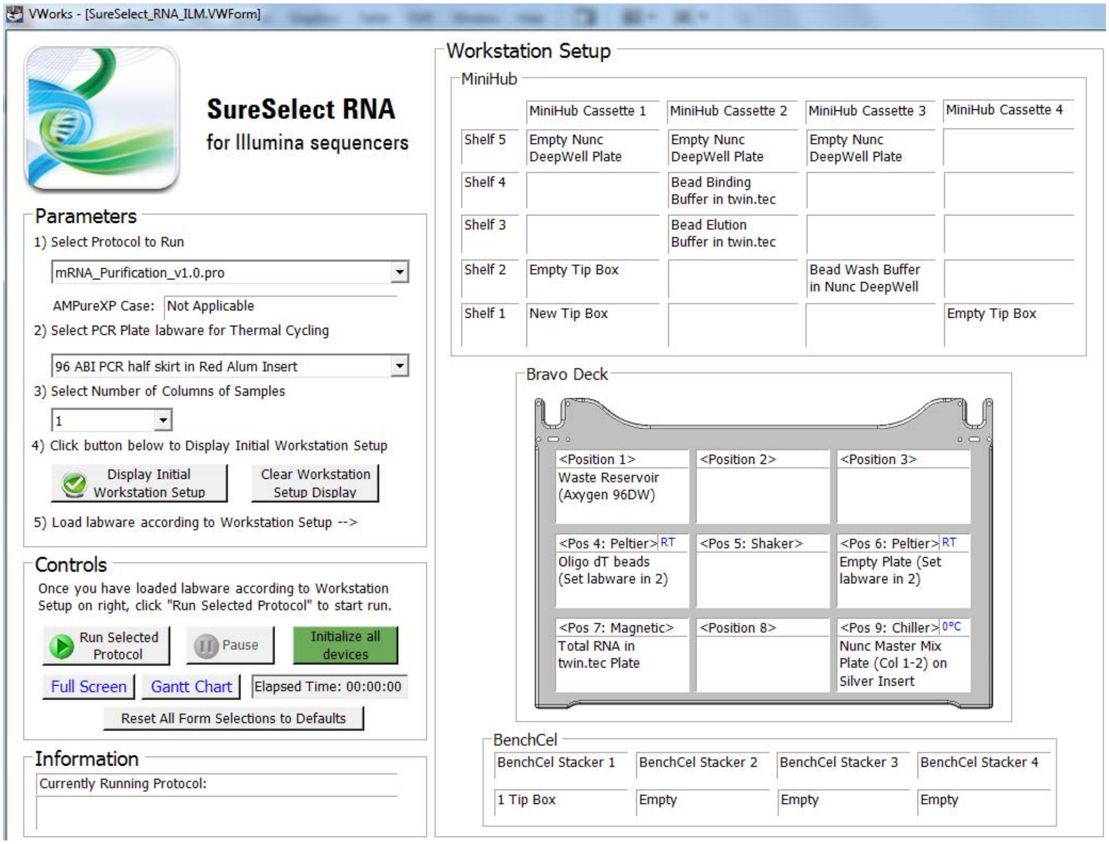
Elapsed Time: 00:00:00

Information

Currently Running Protocol:

1. Open the form using the SureSelect_RNA_ILM.VWForm shortcut on your desktop.
2. Use the drop-down menus on the form to select the appropriate SureSelect workflow step and number of columns of samples for the run.
3. Once all run parameters have been specified on the form, click **Display Initial Workstation Setup.**

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



SureSelect RNA
for Illumina sequencers

Parameters

1) Select Protocol to Run

mRNA_Purification_v1.0.pro

AMPureXP Case: Not Applicable

2) Select PCR Plate labware for Thermal Cycling

96 ABI PCR half skirt in Red Alum Insert

3) Select Number of Columns of Samples

1

4) Click button below to Display Initial Workstation Setup

5) Load labware according to Workstation Setup -->

Controls

Once you have loaded labware according to Workstation Setup on right, click "Run Selected Protocol" to start run.

Elapsed Time: 00:00:00

Information

Currently Running Protocol:

Workstation Setup

MiniHub

	MiniHub Cassette 1	MiniHub Cassette 2	MiniHub Cassette 3	MiniHub Cassette 4
Shelf 5	Empty Nunc DeepWell Plate	Empty Nunc DeepWell Plate	Empty Nunc DeepWell Plate	
Shelf 4		Bead Binding Buffer in twin.tec		
Shelf 3		Bead Elution Buffer in twin.tec		
Shelf 2	Empty Tip Box		Bead Wash Buffer in Nunc DeepWell	
Shelf 1	New Tip Box			Empty Tip Box

Bravo Deck

<Position 1> Waste Reservoir (Axygen 96DW)	<Position 2>	<Position 3>
<Pos 4: Peltier> RT Oligo dT beads (Set labware in 2)	<Pos 5: Shaker>	<Pos 6: Peltier> RT Empty Plate (Set labware in 2)
<Pos 7: Magnetic> Total RNA in twin.tec Plate	<Position 8>	<Pos 9: Chiller> 0°C Nunc Master Mix Plate (Col 1-2) on Silver Insert

BenchCel

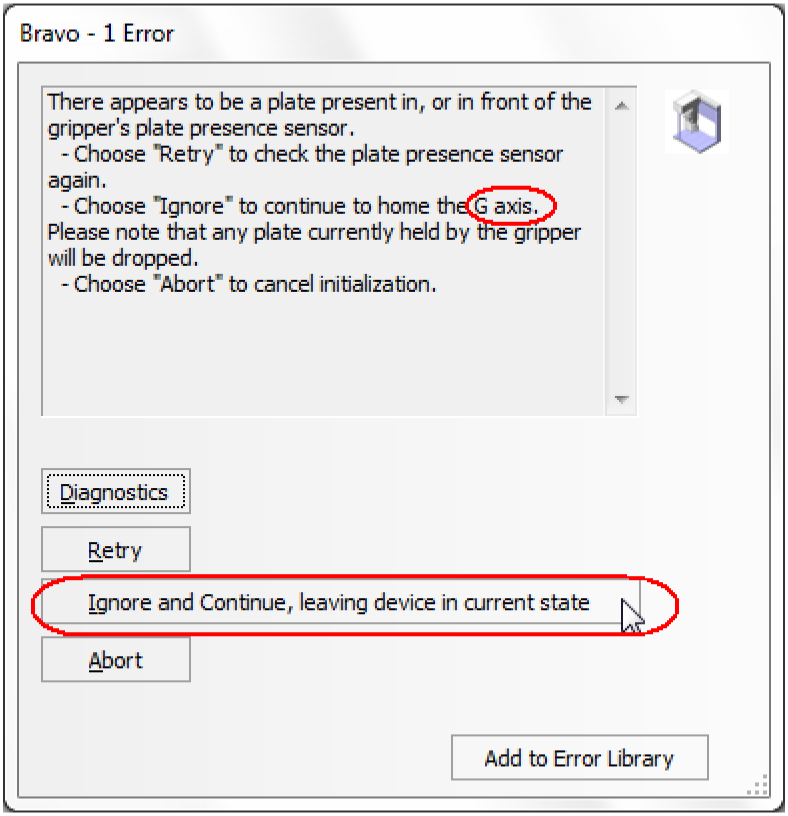
BenchCel Stacker 1	BenchCel Stacker 2	BenchCel Stacker 3	BenchCel Stacker 4
1 Tip Box	Empty	Empty	Empty

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

Error messages encountered at start of run

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

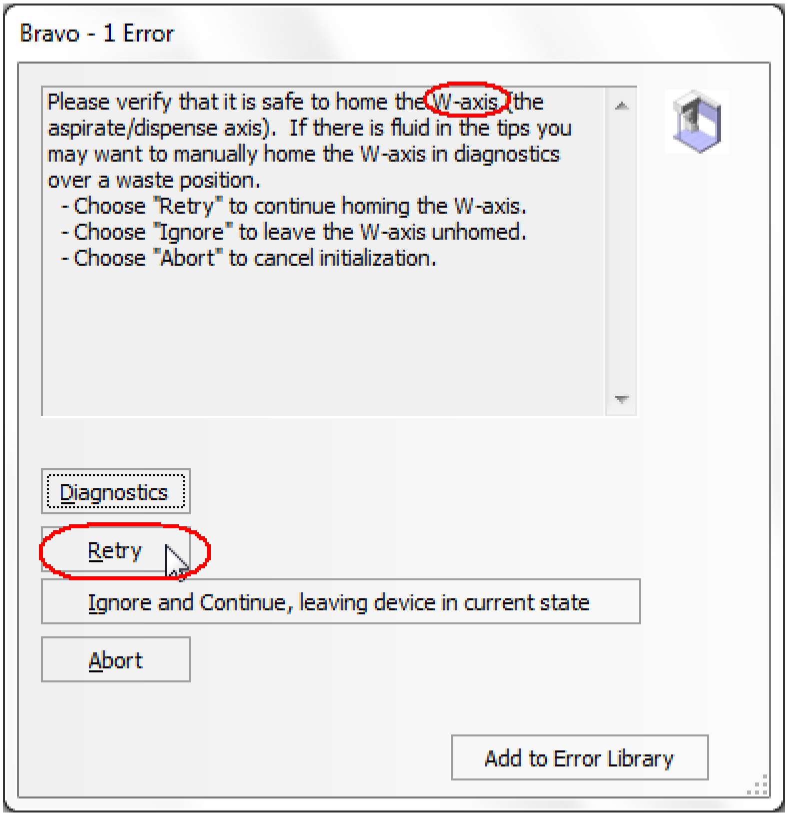
1 If you encounter the G-axis error message shown below, select **Ignore and Continue, leaving device in current state**.



Bravo - 1 Error

There appears to be a plate present in, or in front of the gripper's plate presence sensor.
- Choose "Retry" to check the plate presence sensor again.
- Choose "Ignore" to continue to home the G axis. Please note that any plate currently held by the gripper will be dropped.
- Choose "Abort" to cancel initialization.

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



Please verify that it is safe to home the W-axis (the aspirate/dispense axis). If there is fluid in the tips you may want to manually home the W-axis in diagnostics over a waste position.
- Choose "Retry" to continue homing the W-axis.
- Choose "Ignore" to leave the W-axis unhomed.
- Choose "Abort" to cancel initialization.

Verifying the Simulation setting

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

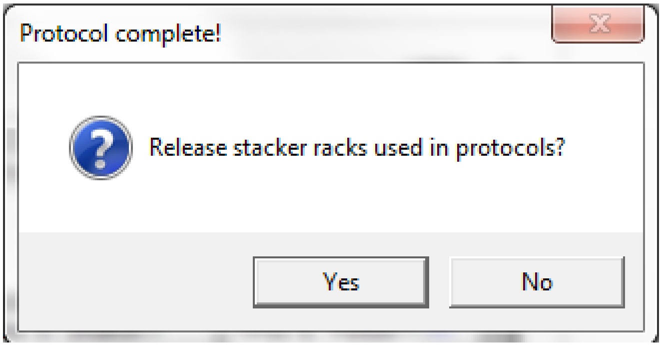
1 Verify that **Simulation is off** is displayed on the status indicator (accessible by clicking **View** > **Control Toolbar**).

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

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

Finishing a protocol or runset

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


Protocol complete!

Release stacker racks used in protocols?

Overview of the SureSelect RNA Library Prep Procedure

Figure 2 summarizes the SureSelect workflow for RNA samples to be sequenced using the Illumina paired-read sequencing platform. For each sample to be sequenced, an individual cDNA library is prepared. The samples are then tagged by PCR with an index sequence. Depending on the capacity of the sequencing platform, up to 48 samples can be pooled and sequenced in a single lane using the multiplex index tags that are provided with the SureSelect Strand-Specific RNA Library Prep kit.

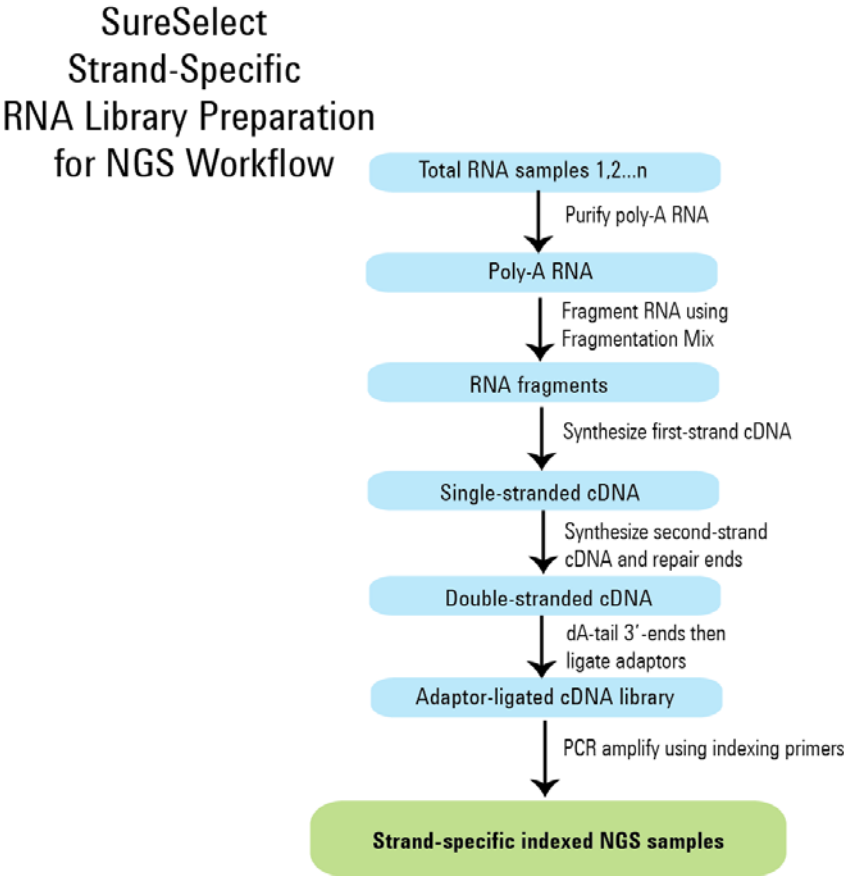


Figure 2 Overall sequencing sample preparation workflow

Table 6 summarizes how the VWorks protocols are integrated into the Strand-Specific RNA Library Prep workflow. See the Sample Preparation chapter for complete instructions for use of the VWorks protocols for sample processing.

Table 6 Overview of VWorks protocols and runsets used during the workflow

Workflow Step	VWorks Protocols Used for Agilent NGS Workstation automation
• Purify poly(A) RNA using oligo(dT) beads • Chemically fragment the poly(A) RNA • Synthesize first-strand cDNA	mRNA_Purification_v1.0.pro
Purify first-strand cDNA using AMPure XP beads	AMPureXP_v1.1.pro:First Strand
• Synthesize second-strand cDNA • Repair DNA ends • Purify end-repaired DNA • dA-tail DNA 3′-ends • Ligate adaptors • Purify adaptor-ligated DNA	LibraryPrep_RNASeq_ILM_v1.1.rst
Amplify adaptor-ligated cDNA library using indexing primers	TranscriptomePCR_ILM_v1.0.pro
Purify indexed library amplicons using AMPure XP beads	AMPureXP_v1.1.pro:Transcriptome PCR
Remove adaptor-dimers using AMPure XP beads	AMPureXP_v1.1.pro:Transcriptome Dimers

Experimental Setup Considerations for Automated Runs

Agilent SureSelect Automated Strand-Specific RNA Library Prep runs may include 1, 2, 3, 4, 6, or 12 columns (equivalent to 8, 16, 24, 32, 48, or 96 wells) of RNA samples to be prepared 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 RNA Samples in 96-well Plates for Automation

- The Agilent NGS Workstation processes samples column-wise beginning at column 1. RNA 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.
- For sample indexing by PCR (see Figure 2), you will need to prepare a separate plate containing the indexing primers. Assign the wells to be indexed with their respective indexing primers during experimental design.

Considerations for Equipment Setup

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

PCR Plate Type Considerations

Automation protocols include several liquid-handling steps in which reagents are dispensed to PCR plates in preparation for transfer to a thermal cycler. For these steps you must specify the PCR plate type to be used on the SureSelect_RNA_ILM.VWForm to allow correct configuration of the liquid handling components for the PCR plate type. Before you begin the automation protocol, make sure that you are using a supported PCR plate type. The PCR plate type to be used in the protocol is specified using the menu below. Vendor and part number information is provided for the supported plate types in Table 8.

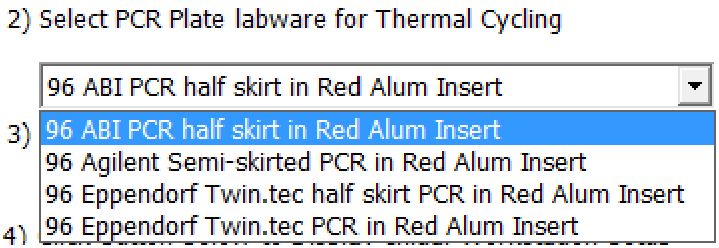


Table 8 Ordering information for supported PCR plates

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

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.

3

Sample Preparation

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

For each sample to be sequenced, individual library preparations are performed in separate wells of a 96-well plate. The samples are then indexed by PCR amplification allowing multiplexing of up to 48 samples for sequencing on Illumina platforms.

Refer to Illumina's protocol *Preparing Samples for Paired-End Sequencing* (p/n 1005361), or the appropriate Illumina protocol for more information.

Step 1. Purify poly(A) RNA/fragment RNA/synthesize first strand cDNA

In this step, automation protocol mRNA_Purification_v1.0.pro is used to complete multiple steps of the RNA Library Preparation workflow. First, poly(A) RNA is purified from total RNA using two serial rounds of binding to oligo(dT) magnetic particles. After purification, the poly(A) RNA is chemically-fragmented to the appropriate size and then is converted to first-strand cDNA.

Total RNA samples containing 50 ng to 4 µg RNA are suitable for the mRNA library preparation automation protocol. Each total RNA sample must be prepared for the run in 25 µL of nuclease-free water.

NOTE For optimal performance, total RNA samples should have an RNA Integrity Number (RIN) of 8 or more, based on analysis using Agilent's 2100 Bioanalyzer.

Consider preparing an additional sequencing library in parallel, using a high-quality control RNA sample, such as Agilent QPCR Human Reference Total RNA (p/n 750500). Use of this control is especially recommended during the first run of the protocol, to verify that all protocol steps are being successfully performed. Routine use of this control is helpful for any required troubleshooting, in order to differentiate any performance issues related to RNA input from other factors.

A workstation operator must be present during this automation protocol to transfer plates between the workstation, which completes most liquid handling steps, and the thermal cycler, which is used for several incubation steps. In addition, the operator must prepare and dispense a master mix immediately before it is used in the automation protocol (see step 30 on page 48).

Prepare the workstation

1. Open the SureSelect setup form using the SureSelect_RNA_ILM.VWForm shortcut on your desktop.
2. Log in to the VWorks software.

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

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

Prepare reagents for the run

5 Bring the reagents listed in Table 9 to room temperature and thaw the reagents listed in Table 10 on ice.

Table 9 Reagents brought to room temperature before use in protocol

Storage Location	Kit Component	Where Used in Protocol
SureSelect Poly-A Selection Module[*], 4°C	Oligo(dT) Microparticles (bottle)	page 35
	Bead Washing Buffer[†] (bottle)	page 35
	Bead Elution Buffer[‡] (bottle)	page 35
	Bead Binding Buffer[**] (bottle)	page 35

[*] May also be labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 2.*

[†] May also be labeled as *RNA Seq Bead Washing Buffer.*

[‡] May also be labeled as *RNA Seq Bead Elution Buffer.*

[**] May also be labeled as *RNA Seq Bead Binding Buffer.*

Table 10 Reagents thawed and held on ice before use in protocol

Storage Location	Kit Component	Where Used in Protocol
SureSelect RNA Library Prep, ILM (Pre PCR)[*], –20°C	Fragmentation Mix[†] (bottle)	page 36
	First Strand Master Mix[‡] (tube with orange cap)	page 49

[*] May also be labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 1.*

[†] May also be labeled as *RNA Seq Fragmentation Mix.*

[‡] May also be labeled as *RNA Seq First Strand Master Mix.*

6 Locate or prepare a stock solution of 4 µg/µL Actinomycin D in DMSO. When first prepared, aliquot the stock solution into single-use volumes (typically 3 µL) and store the aliquots at –20°C, protected from light. Do not subject the aliquots to multiple freeze-thaw cycles. The aliquots may be stored for up to one year before use in the library preparation protocol.

A 3-µL aliquot of this DMSO stock solution will be used on page 48 to prepare a fresh dilution of 120 ng/µL Actinomycin D in water for the run.

CAUTION To ensure strand specificity, take care to follow the storage instructions above for the 4 µg/µL Actinomycin D in DMSO stock solution, including the one-year maximum storage duration.

Prepare the RNA samples source plate

7 Place 25 µL of each RNA sample (0.05–4 µg RNA in nuclease-free water) into the wells of a 96-well Eppendorf twin.tec plate. Load samples into the plate column-wise, in well order A1 to H1, then A2 to H2, ending with A12 to H12 for processing on the Agilent NGS Workstation.

NOTE SureSelect Strand-Specific RNA Library Prep runs may include 1, 2, 3, 4, 6, or 12 columns of the plate. See Using the Agilent NGS Workstation for SureSelect RNA Library Preparation for additional sample placement considerations.

Prepare the oligo(dT) beads and mRNA purification source plates

When preparing each of the source plates below, add the indicated amount of reagent to wells of the source plate corresponding to the total RNA sample wells in step 7 above. For example, for 3-column runs, fill source well plate wells A1 to H3, but leave wells A4 to H12 empty.

8 Prepare the oligo(dT) beads source plate.

  a Vortex the Oligo(dT) Microparticles until the suspension appears homogeneous and consistent in color. If bead aggregates are still present after vortexing, mix thoroughly by pipetting up and down until the suspension appears homogeneous.

  b In a PCR plate that is compatible with the thermal cycler to be used in the run, place 25 µL of the homogeneous Oligo(dT) bead suspension into each well to be used for sample purification.

9 Prepare the Bead Binding Buffer source plate. Place 30 µL of Bead Binding Buffer into wells of a 96-well Eppendorf twin.tec plate. Fill each well that corresponds to an RNA sample well.

10 Prepare the Bead Elution Buffer source plate. Place 30 µL of Bead Elution Buffer into wells of a 96-well Eppendorf twin.tec plate. Fill each well that corresponds to an RNA sample well.

11 Prepare the Bead Wash Buffer source plate. Place 410 µL of Bead Washing Buffer into wells of a Nunc DeepWell plate. Fill each well that corresponds to an RNA sample well.

Prepare the master mix source plate

12 Prepare the master mix source plate by adding the appropriate volume of Fragmentation Mix (see Table 11) to all wells of Column 1 of a Nunc DeepWell plate. The configuration of the source plate is shown in Figure 3.

Table 11 Preparation of the Master Mix Source Plate for mRNA_Purification_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
Fragmentation Mix	Column 1 (A1-H1)	28.5 µL	47.5 µL	66.5 µL	85.5 µL	123.5 µL	247.0 µL

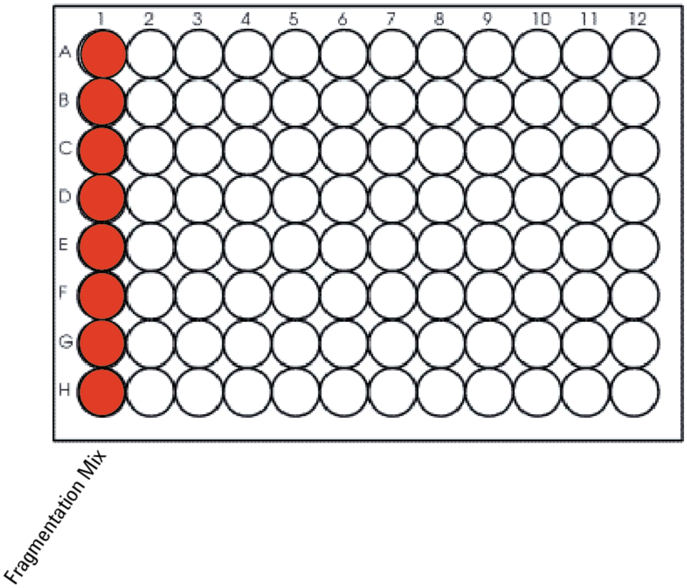


Figure 3 Initial configuration of master mix source plate for mRNA_Purification_v1.0.pro

Load the Agilent NGS Workstation

13 Load the Labware MiniHub according to Table 12, using the plate orientations shown in Figure 4.

Table 12 Initial MiniHub configuration for mRNA_Purification_v1.0.pro

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	Empty Nunc DeepWell plate	Empty Nunc DeepWell plate	Empty
Shelf 4	Empty	Bead Binding Buffer in twin.tec plate	Empty	Empty
Shelf 3	Empty	Bead Elution Buffer in twin.tec plate	Empty	Empty
Shelf 2	Empty tip box	Empty	Bead Wash Buffer in Nunc DeepWell plate	Empty
Shelf 1 (Bottom)	New tip box	Empty	Empty	Empty tip box

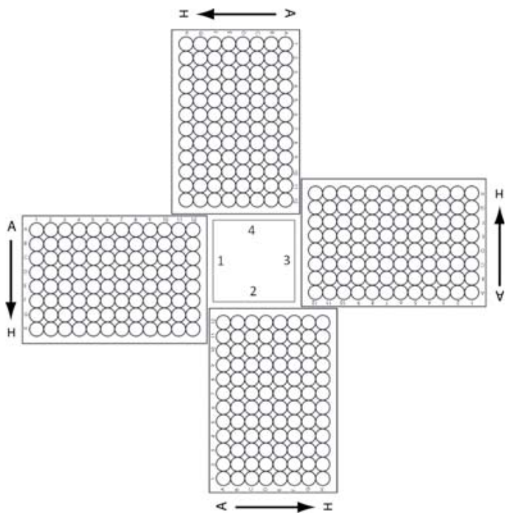


Figure 4 Agilent Labware MiniHub plate orientation. For Thermo Scientific reservoirs, place the notched corner facing the center of the hub.

14 Load the Bravo deck according to Table 13.

Table 13 Initial Bravo deck configuration for mRNA_Purification_v1.0.pro

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
4	Oligo(dT) beads in PCR plate seated on red insert (PCR plate type must be specified on setup form under step 2)
6	Empty PCR plate seated on red insert (PCR plate type must be specified on setup form under step 2)
7	Total RNA samples in twin.tec plate
9	Master Mix Source Plate seated on silver insert (Nunc DeepWell; see Figure 3 on page 36 for column content)

15 Load the BenchCel Microplate Handling Workstation according to Table 14.

Table 14 Initial BenchCel configuration for mRNA_Purification_v1.0.pro

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	Empty	Empty	Empty
2	2 Tip boxes	Empty	Empty	Empty
3	3 Tip boxes	Empty	Empty	Empty
4	3 Tip boxes	Empty	Empty	Empty
6	5 Tip boxes	Empty	Empty	Empty
12	9 Tip boxes	Empty	Empty	Empty

Run VWorks protocol mRNA_Purification_v1.0.pro

16 On the SureSelect setup form, under **Select Protocol to Run**, select **mRNA_Purification_v1.0.pro.**

17 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate that was loaded on Bravo deck positions 4 and 6. The plate type selected must be compatible with the thermal cycler to be used for incubation steps during the protocol.

NOTE

During run setup, be sure to use the plate type selected from this menu at positions 4 and 6 of the Bravo deck. In addition, when the workstation issues prompts to add plates to postion 4 or 6 during the run, use only the same PCR plate type specified here.

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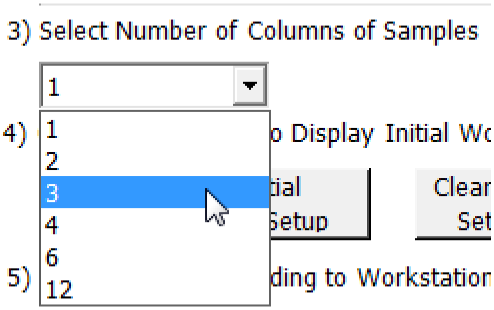
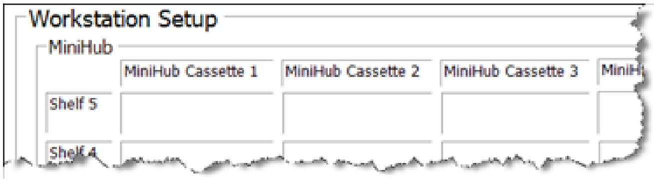
18 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.



19 Click **Display Initial Workstation Setup**.

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



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

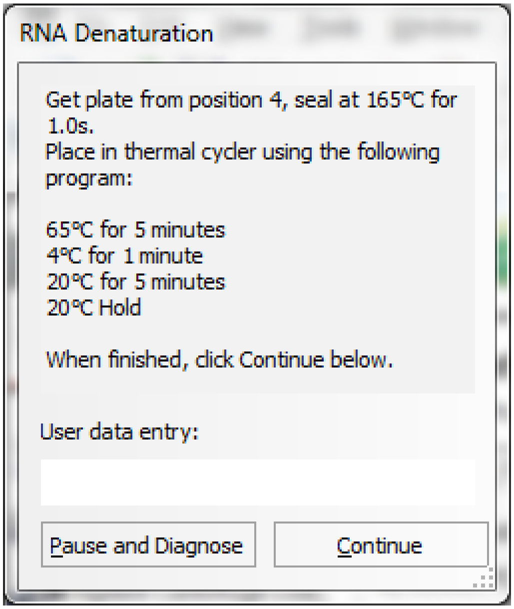
NOTE

If workstation devices do not respond when you start the run, but activity is recorded in the Log, verify that VWorks is not running in Simulation mode. See page 25 for more information.

Running the mRNA_Purification_v1.0.pro protocol takes approximately 90 minutes, including four incubation periods on the thermal cycler.

During the automation protocol run, a workstation operator must be present to transfer plates between the workstation and thermal cycler when prompted, as detailed on the following pages.

22 When the workstation has finished combining the RNA samples with the oligo(dT) beads, you will be prompted by VWorks as shown below.



Get plate from position 4, seal at 165°C for 1.0s.
Place in thermal cycler using the following program:

65°C for 5 minutes
4°C for 1 minute
20°C for 5 minutes
20°C Hold

When finished, click Continue below.

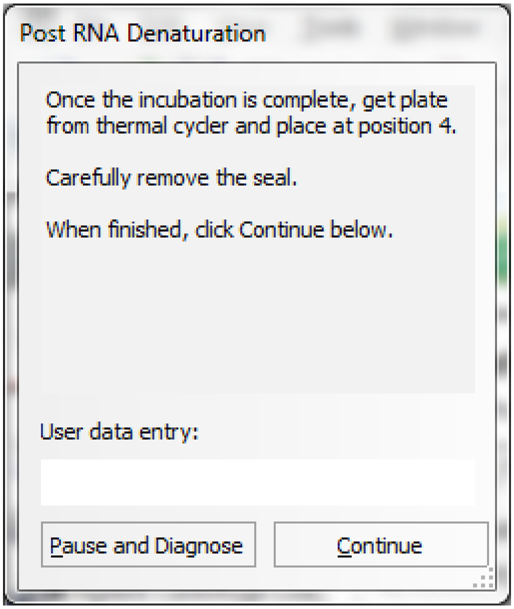
User data entry:

- **a** Remove the plate from position 4 of the Bravo deck and seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 seconds.
- **b** Briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid without pelleting the beads.
- **c** Transfer the PCR plate to a thermal cycler (with the heated lid ON) and run the RNA denaturation + bead binding program shown in Table 15. After transferring the plate, click **Continue** on the VWorks screen.

Table 15 Thermal cycler program for RNA denaturation and RNA-bead binding

Step	Temperature	Time
Step 1	65°C	5 minutes
Step 2	4°C	1 minute
Step 3	20°C	5 minutes
Step 4	20°C	Hold

23 After the thermal cycler reaches the 20°C Hold step, and when prompted by the dialog below, transfer the RNA sample plate to position 4 of the Bravo deck, seated in the red insert. Carefully unseal the plate, then click **Continue**.



Post RNA Denaturation

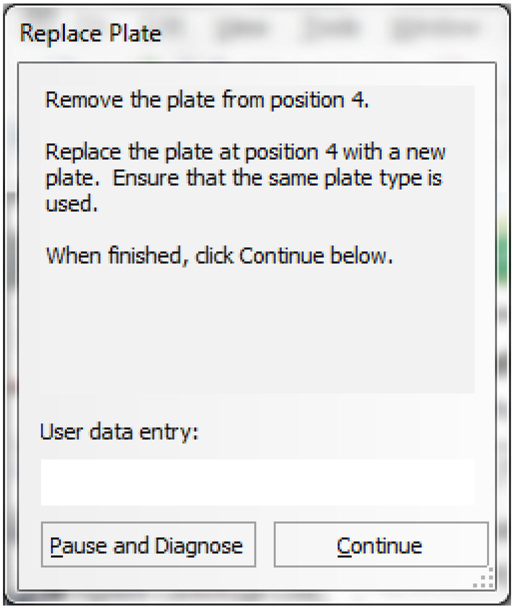
Once the incubation is complete, get plate from thermal cycler and place at position 4.

Carefully remove the seal.

When finished, click Continue below.

User data entry:

24 When the workstation has finished collecting and washing the bead-bound RNA samples, you will be prompted by VWorks as shown below.



Replace Plate

Remove the plate from position 4.

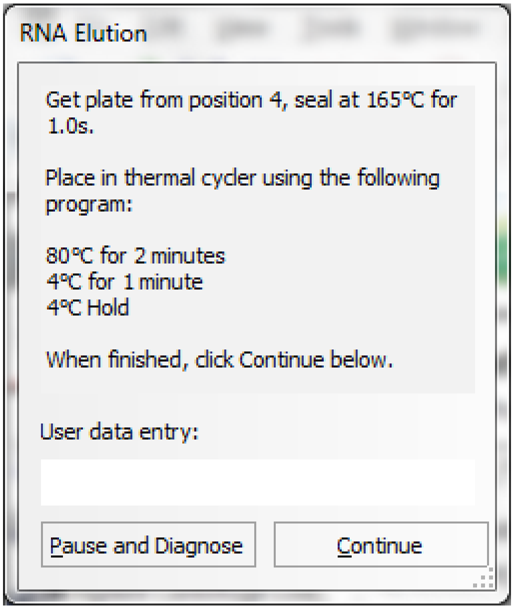
Replace the plate at position 4 with a new plate. Ensure that the same plate type is used.

When finished, click Continue below.

User data entry:

- **a** Remove and discard the PCR plate from position 4 of the Bravo deck.
- **b** Place a fresh PCR plate at position 4, seated in the red insert. The PCR plate type added here must be the same plate type as the one removed and as was specified during the run setup.
- **c** After positioning the plate, click **Continue** on the VWorks screen.

25 When the workstation has finished collecting and washing the bead-bound RNA samples, you will be prompted to transfer the plate to the thermal cycler for the RNA Elution step as shown below.



Get plate from position 4, seal at 165°C for 1.0s.

Place in thermal cycler using the following program:

80°C for 2 minutes
4°C for 1 minute
4°C Hold

When finished, click Continue below.

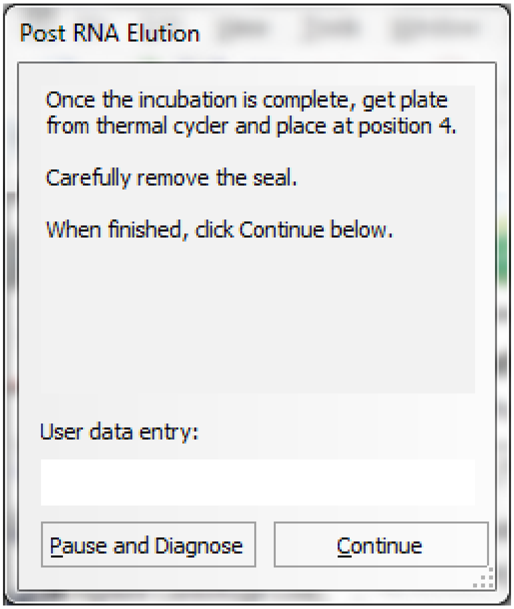
User data entry:

- **a** Remove the plate from position 4 of the Bravo deck and seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 seconds.
- **b** Briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid without pelleting the beads.
- **c** Transfer the PCR plate to a thermal cycler (with the heated lid ON) and run the RNA elution program shown in Table 16. After transferring the plate, click **Continue** on the VWorks screen.

Table 16 Thermal cycler program for RNA elution

Step	Temperature	Time
Step 1	80°C	2 minutes
Step 2	4°C	1 minute
Step 3	4°C	Hold

26 After the thermal cycler reaches the 4°C Hold step and when prompted by the dialog below, transfer the RNA sample plate to position 4 of the Bravo deck, seated in the red insert. Carefully unseal the plate, then click **Continue**.


Post RNA Elution

Once the incubation is complete, get plate from thermal cycler and place at position 4.

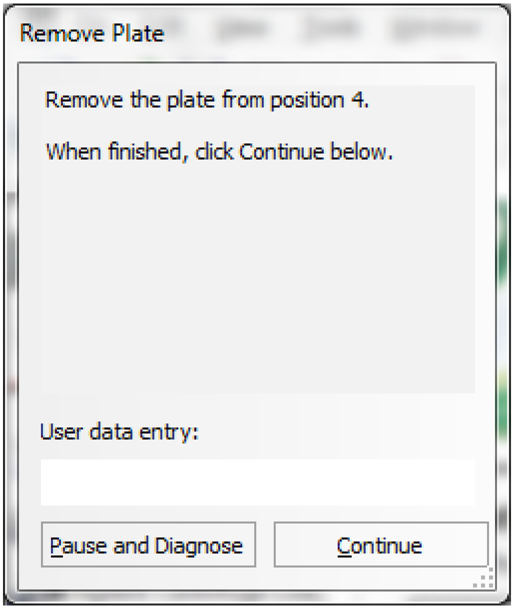
Carefully remove the seal.

When finished, click Continue below.

User data entry:

27 The workstation adds Bead Binding Buffer to the eluted RNA samples and then holds the samples at room temperature for 5 minutes to allow the poly(A) RNA to re-bind the beads.

28 When the workstation has finished the collecting and washing the bound RNA samples in this second round of purification, you will be prompted by VWorks as shown below. Remove and discard the PCR plate from position 4, then click **Continue**.



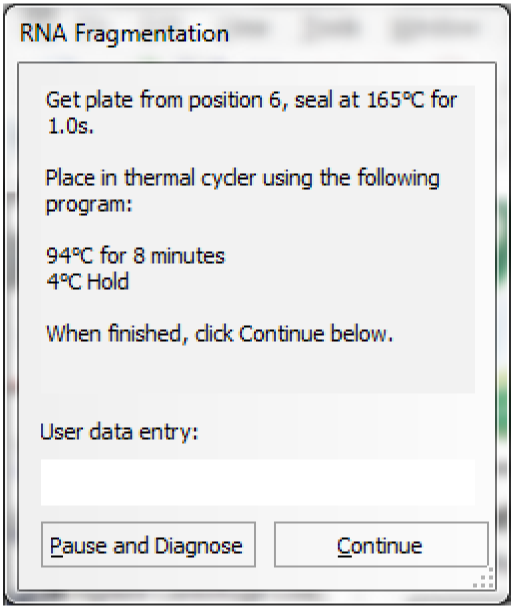
Remove Plate

Remove the plate from position 4.

When finished, click Continue below.

User data entry:

29 The workstation adds Fragmentation Mix to the bead-bound RNA samples in preparation for the RNA fragmentation step. When the workstation has finished, you will be prompted by VWorks as shown below.



RNA Fragmentation

Get plate from position 6, seal at 165°C for 1.0s.

Place in thermal cycler using the following program:

94°C for 8 minutes
4°C Hold

When finished, click Continue below.

User data entry:

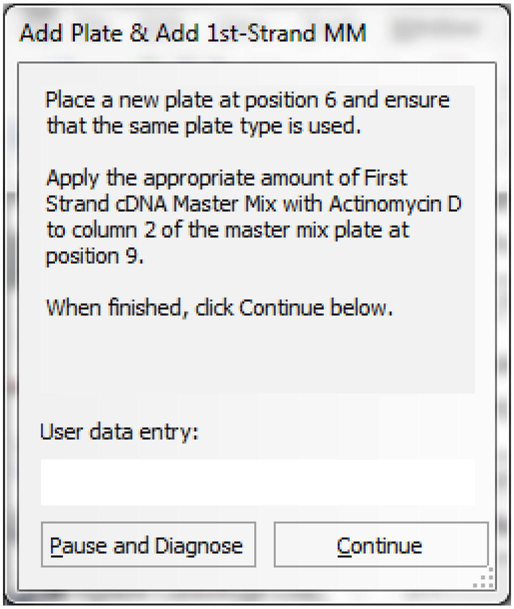
- **a** Remove the plate from position 6 of the Bravo deck and seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 seconds.
- **b** Briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
- **c** Transfer the PCR plate to a thermal cycler (with the heated lid ON) and run the RNA fragmentation program shown in Table 17. After transferring the plate, click **Continue** on the VWorks screen.

Table 17 Thermal cycler program for RNA fragmentation

Step	Temperature	Time
Step 1	94°C	8 minutes
Step 2	4°C	Hold

30 During the 8-minute incubation step, prepare the reagents and workstation for first-strand cDNA synthesis:

  a When prompted by the dialog below, place a fresh PCR plate (use the plate type specified during the run setup) at position 6, seated in the red insert. Proceed immediately to step b, below.



Add Plate & Add 1st-Strand MM

Place a new plate at position 6 and ensure that the same plate type is used.

Apply the appropriate amount of First Strand cDNA Master Mix with Actinomycin D to column 2 of the master mix plate at position 9.

When finished, click Continue below.

User data entry:

  b Prepare a fresh 120 ng/µL Actinomycin D dilution in water from a stock solution of 4 µg/µL Actinomycin D in DMSO, according to Table 18.

CAUTION To ensure strand-specificity, you must prepare the 120 ng/µL Actinomycin D dilution in water immediately before use in step d, below.

Table 18 Preparation of 120 ng/µl Actinomycin D

Reagent	Volume for up to 12-column run (includes excess)
Actinomycin D (4 µg/µl in DMSO)	3 µL
Nuclease-free water	97 µL
Total	**100 µL**

c Vortex the thawed vial of First Strand Master Mix 5 seconds at high speed to ensure homogeneity.

CAUTION SureSelect RNA Library Prep master mixes are viscous. Mix thoroughly by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining the master mix with other solutions. Pipetting up and down is not sufficient to mix this reagent.

d Prepare the appropriate amount of First Strand Master Mix + Actinomycin D mixture, according to the table below. Mix by vortexing, then spin briefly and keep on ice.

Table 19 Preparation of First Strand Master Mix/Actinomycin D 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
First Strand Master Mix	8.0 µL	98.4 µL	196.8 µL	262.4 µL	360.8 µL	492.0 µL	918.4 µL
Actinomycin D (120 ng/µl in H_2O)	0.5 µL	6.2 µL	12.3 µL	16.4 µL	22.6 µL	30.8 µL	57.4 µL
Total Volume	**8.5 µL**	**104.6 µL**	**209.1 µL**	**278.8 µL**	**383.4 µL**	**522.8 µL**	**975.8 µL**

e Add the volume listed in Table 20 of the First Strand Master Mix + Actinomycin D mixture to column 2 of the Master Mix source plate at position 9 of the Bravo deck. The final configuration of the source plate is shown in Figure 13. After adding the master mix to the source plate, click **Continue** on the VWorks screen.

Table 20 Preparation of the Master Mix Source Plate for mRNA_Purification_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
First Strand Master Mix + Actinomycin D mixture	Column 2 (A2-H2)	12.0 µL	25.1 µL	33.8 µL	46.9 µL	64.3 µL	120.9 µL

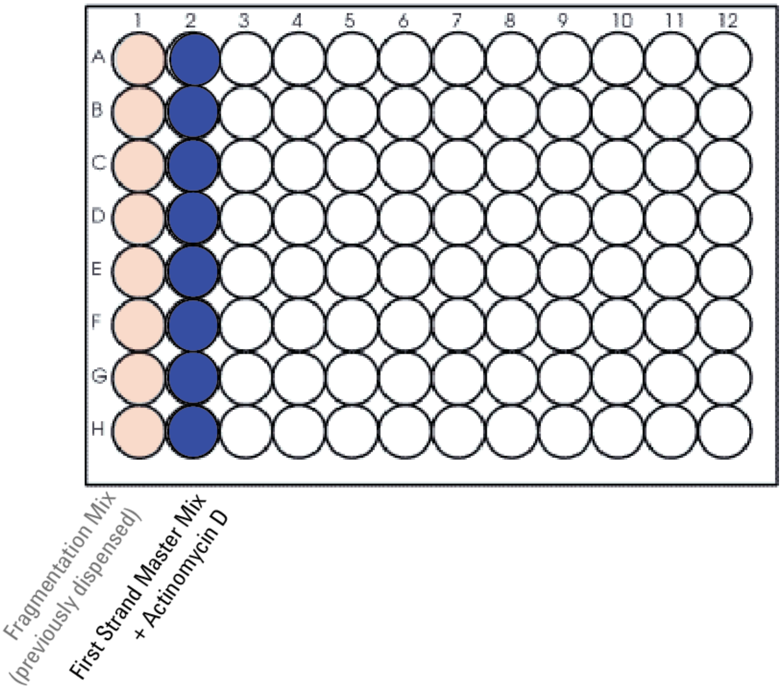
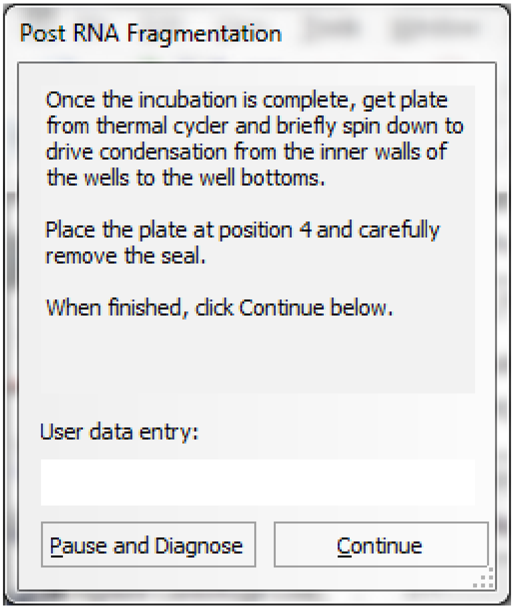


Figure 5 Final configuration of master mix source plate for `mRNA_Purification_v1.0.pro`

31 With the RNA sample plate still on the thermal cycler, the workstation prepares the remaining components for first-strand cDNA synthesis. When the workstation has finished, you will be prompted by VWorks as shown below.



Post RNA Fragmentation

Once the incubation is complete, get plate from thermal cycler and briefly spin down to drive condensation from the inner walls of the wells to the well bottoms.

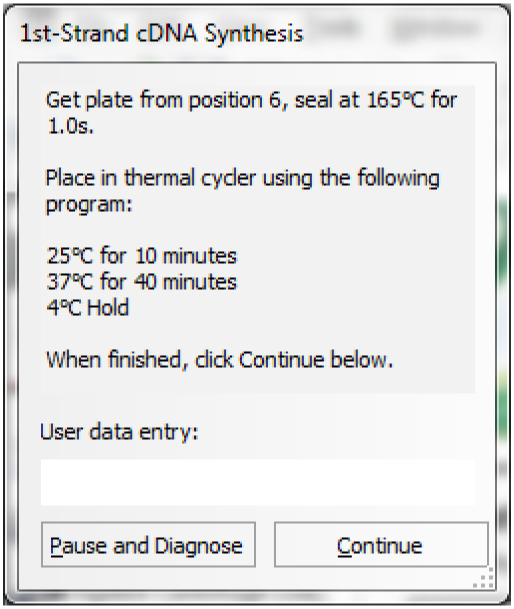
Place the plate at position 4 and carefully remove the seal.

When finished, click Continue below.

User data entry:

- **a** After the thermal cycler reaches the 4°C Hold step for the RNA fragmentation program (Table 17), remove the plate from the thermal cycler and briefly spin in a centrifuge or mini-plate spinner to collect the liquid.
- **b** Place the RNA sample plate on position 4 of the Bravo deck, seated in the red insert.
- **c** Carefully unseal the plate, then click **Continue.**

32 The workstation removes the fragmented RNA samples from the bead-containing wells and combines the samples with First Strand Master Mix + Actinomycin D. When the workstation has finished, you will be prompted by VWorks as shown below.



1st-Strand cDNA Synthesis

Get plate from position 6, seal at 165°C for 1.0s.

Place in thermal cycler using the following program:

25°C for 10 minutes
37°C for 40 minutes
4°C Hold

When finished, click Continue below.

User data entry:

- **a** Remove the 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 1.0 seconds.
- **b** Briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
- **c** Transfer the PCR plate to a thermal cycler (with the heated lid ON) and run the first-strand cDNA synthesis program shown in Table 21. After transferring the plate, click **Continue** on the VWorks screen.

Table 21 Thermal cycler program for first-strand cDNA synthesis

Step	Temperature	Time
Step 1	25°C	10 minutes
Step 2	37°C	40 minutes
Step 3	4°C	Hold

Step 2. Purify first-strand cDNA using AMPure XP beads

In this step, the Agilent NGS Workstation transfers AMPure XP beads and first-strand cDNA samples to a Nunc DeepWell plate and then collects and washes the bead-bound DNA.

Prepare the workstation and reagents

1. Leave tip boxes on shelves 1 and 2 in casette 1 of the Labware MiniHub from the previous mRNA_Purification_v1.0.pro run. Otherwise, clear the remaining positions of the MiniHub and BenchCel of plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
5. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
6. Prepare a Nunc DeepWell source plate for the beads by adding 51 µL of homogeneous AMPure XP beads per well, for each well to be processed.
7. Prepare a Thermo Scientific reservoir containing 15 mL of nuclease-free water.
8. Prepare a separate Thermo Scientific reservoir containing 45 mL of freshly-prepared 70% ethanol.

9 Load the Labware MiniHub according to Table 22, using the plate orientations shown in Figure 4.

Table 22 Initial MiniHub configuration for AMPureXP_v1.1.pro:First Strand

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	Empty	Empty	Empty
Shelf 4	Empty	Empty	Empty	Empty
Shelf 3	Empty	Empty Eppendorf twin.tec Plate	Empty	Empty
Shelf 2	Waste tip box (retained from mRNA_Purification protocol)*	Nuclease-free water reservoir from step 7	AMPure XP beads in Nunc DeepWell plate from step 6	Empty
Shelf 1 (Bottom)	Clean tip box (retained from mRNA_Purification protocol)*	70% ethanol reservoir from step 8	Empty	Empty tip box

* The tip boxes retained in Cassette 1 are not shown on the VWorks Workstation Setup table. These tip boxes are not used in AMPureXP_v1.1.pro:First Strand but are used in a later protocol. This labware should be retained in the MiniHub to ensure that empty and full tip positions are properly defined for the subsequent protocol.

10 Load the Bravo deck according to Table 23.

Table 23 Initial Bravo deck configuration for AMPureXP_v1.1.pro:First Strand

Location	Content
1	Empty waste reservoir (Axygen 96 Deep Well Plate, square wells)
9	First-strand cDNA samples in PCR plate seated on red insert (PCR plate type must be specified on setup form under step 2)

11 Load the BenchCel Microplate Handling Workstation according to Table 24.

Table 24 Initial BenchCel configuration for AMPureXP_v1.1.pro:First Strand

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

Run VWorks protocol AMPureXP_v1.1.pro:First Strand

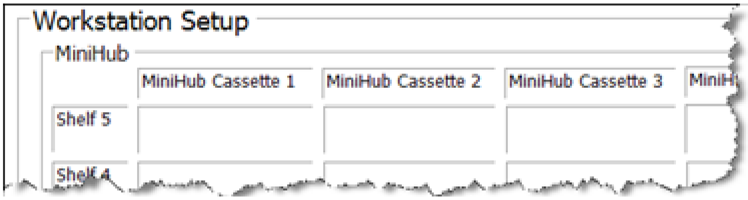
12 On the SureSelect setup form, under **Select Protocol to Run**, select **AMPureXP_v1.1.pro:First Strand.**

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

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

15 Click **Display Initial Workstation Setup**.

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



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

Running the AMPureXP_v1.1.pro:First Strand protocol takes approximately 45 minutes. During this time, you can prepare the purification reagents for the Library Prep automation protocol, as described on page 58.

Once the AMPureXP_v1.1.pro:First Strand protocol is complete, the purified cDNA samples are located in the Eppendorf plate at position 7 of the Bravo deck. Proceed immediately to “Step 3. Prepare cDNA libraries for Illumina sequencing” on page 57.

Step 3. Prepare cDNA libraries for Illumina sequencing

This step is automated using the LibraryPrep_RNASeq_ILM_v1.1.rst runset. During the runset, the Agilent NGS Workstation completes second-strand cDNA library synthesis and the end modification steps required for sequencing, including end-repair, A-tailing, and adaptor ligation. After certain modification steps, the Agilent NGS Workstation purifies the prepared cDNA using AMPure XP beads.

This step uses the SureSelect RNA Reagent Kit components listed in Table 25 in addition to the purification reagents prepared for use on page 58. Thaw each reagent vial and keep on ice. Vortex each vial for 5 seconds at high speed to mix before use.

Table 25 Reagents for automation runset LibraryPrep_RNASeq_ILM_v1.1.rst

Storage Location	Kit Component	Where Used in Protocol
SureSelect RNA Library Prep, ILM (Pre PCR)[*], –20°C	Second Strand Enzyme Mix[†] (bottle)	page 59
	Second Strand Oligo Mix[‡] (tube with yellow cap)	page 59
	SureSelect Ligation Master Mix (tube with purple cap)	page 59
	SureSelect Oligo Adaptor Mix (tube with blue cap)	page 59
	dA Tailing Master Mix[**] (bottle)	page 59

[*] May also be labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 1.*

[†] May also be labeled as *RNA Seq Second Strand + End Repair Enzyme Mix.*

[‡] May also be labeled as *RNA Seq Second Strand + End Repair Oligo Mix.*

[**] May also be labeled as *RNA Seq dA Tailing Master Mix.*

Prepare the workstation

1. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
2. Leave tip boxes on shelves 1 and 2 in cassette 1 of the Labware MiniHub from the previous mRNA_Purification_v1.0.pro run. Otherwise, clear the remaining MiniHub and BenchCel positions of plates and tip boxes.

3. Pre-set the temperature of Bravo deck position 4 to 14°C and of position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. On the control touchscreen, Bravo deck positions 4 corresponds to CPAC 2, position 1, while deck position 6 corresponds to CPAC 2, position 2.

Prepare the purification reagents

4. Verify that the AMPure XP bead suspension is at room temperature. *Do not freeze the beads at any time.*
5. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
6. Prepare a Nunc DeepWell source plate for the beads by adding 160 µL of homogeneous AMPure XP beads per well, for each well to be processed.
7. Prepare a Thermo Scientific reservoir containing 20 mL of nuclease-free water.
8. Prepare a separate Thermo Scientific reservoir containing 150 mL of freshly-prepared 70% ethanol.

Prepare the master mix source plate

9 Vortex the thawed vials of Second Strand Enzyme Mix, SureSelect Ligation Master Mix, and dA Tailing Master Mix for 5 seconds at high speed to ensure homogeneity.

CAUTION SureSelect RNA Library Prep master mixes are viscous. Mix thoroughly by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining the master mix with other solutions. Pipetting up and down is not sufficient to mix these reagents.

10 Prepare the appropriate amount of Second Strand + End Repair Master Mix according to Table 26 below.

Table 26 Preparation of Second Strand + End Repair Master Mix for LibraryPrep_RNASeq_ILM_v1.1.rst

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Second Strand Enzyme Mix	25.0 µL	307.5 µL	615 µL	820 µL	1127.5 µL	1640 µL	3075 µL
Second Strand Oligo Mix	5.0 µL	61.5 µL	123 µL	164 µL	225.5 µL	328 µL	615 µL
Total Volume	**30 µL**	**369 µL**	**738 µL**	**984 µL**	**1353 µL**	**1968 µL**	**3690 µL**

11 Prepare the appropriate amount of Adaptor Ligation Master Mix, containing the SureSelect Ligation Master Mix and the adaptors, according to Table 27 below.

Table 27 Preparation of Adaptor Ligation Master Mix for LibraryPrep_RNASeq_ILM_v1.1.rst

Reagent	Volume for 1 Library	Volume for 1 Column	Volume for 2 Columns	Volume for 3 Columns	Volume for 4 Columns	Volume for 6 Columns	Volume for 12 Columns
Nuclease-free water	2.5 µL	30.8 µL	61.5 µL	82.0 µL	112.8 µL	164.0 µL	307.5 µL
SureSelect Ligation Master Mix	5.0 µL	61.5 µL	123.0 µL	164.0 µL	225.5 µL	328.0 µL	615.0 µL
SureSelect Oligo Adaptor Mix	5.0 µL	61.5 µL	123.0 µL	164.0 µL	225.5 µL	328.0 µL	615.0 µL
Total Volume	**12.5 µL**	**153.8 µL**	**307.5 µL**	**410.0 µL**	**563.8 µL**	**820.0 µL**	**1537.5 µL**

12 Using the same Nunc DeepWell master mix source plate that was used for the mRNA_Purification_v1.0.pro run, prepare the Library Prep master mix source plate. Add the volumes indicated in Table 28 of each master mix to all wells of the indicated column of the plate. Keep the master mixes on ice during the aliquoting steps. The final configuration of the master mix source plate is shown in Figure 13.

Table 28 Preparation of the Master Mix Source Plate for LibraryPrep_RNASeq_ILM_v1.1.rst

Master Mix Solution	Position on Source Plate	Volume of Master Mix added per Well of Nunc Deep Well Source Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Second Strand + End Repair Master Mix (Table 26)	Column 3 (A3-H3)	42.4 µL	88.5 µL	119.3 µL	165.4 µL	242.3 µL	457.5 µL
dA Tailing Master Mix	Column 4 (A4-H4)	30.0 µL	50.0 µL	70.0 µL	90.0 µL	130.0 µL	260.0 µL
Adaptor Ligation Master Mix (from Table 27)	Column 5 (A5-H5)	17.7 µL	36.9 µL	49.7 µL	68.9 µL	100.9 µL	190.6 µL

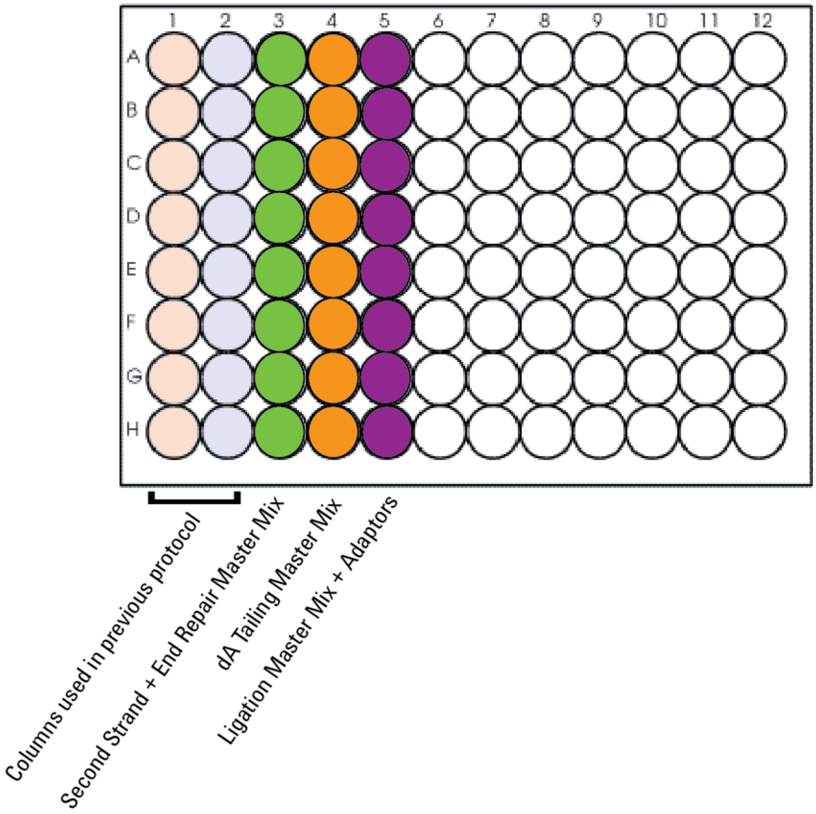


Figure 6 Configuration of the master mix source plate for LibraryPrep_RNASeq_ILM_v1.1.rst

NOTE

If you are using a new DeepWell plate for the Library Prep Master Mix source plate, leave columns 1 and 2 empty and add the PCR Master Mix to columns 3 to 5 of the new plate.

---

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

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

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

Load the Agilent NGS Workstation

15 Load the Labware MiniHub according to Table 29, using the plate orientations shown in Figure 4.

Table 29 Initial MiniHub configuration for LibraryPrep_RNASeq_ILM_v1.1.rst

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty	Empty Nunc DeepWell plate	Empty Nunc DeepWell plate	Empty
Shelf 4	Empty	Empty Eppendorf twin.tec plate	Empty Eppendorf twin.tec plate	Empty
Shelf 3	Empty	Empty	Empty Eppendorf twin.tec plate	Empty
Shelf 2	Waste tip box (retained from mRNA_Purification protocol)	Nuclease-free water reservoir from step 7	AMPure XP beads in Nunc DeepWell plate from step 6	Empty
Shelf 1 (Bottom)	Clean tip box (retained from mRNA_Purification protocol)	70% ethanol reservoir from step 8	Empty	Empty tip box

16 Load the Bravo deck according to Table 30.

Table 30 Initial Bravo deck configuration for LibraryPrep_RNASeq_ILM_v1.1.rst

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

17 Load the BenchCel Microplate Handling Workstation according to Table 31.

Table 31 Initial BenchCel configuration for LibraryPrep_RNASeq_ILM_v1.1.rst

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

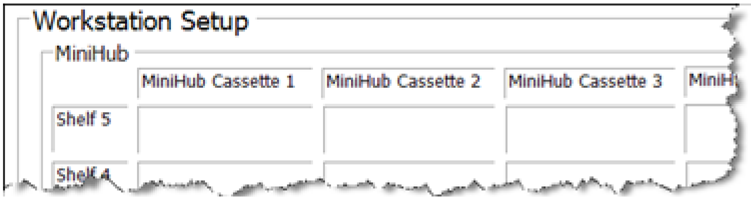
Run VWorks runset LibraryPrep_RNASeq_ILM_v1.1.rst

18 On the SureSelect setup form, under **Select Protocol to Run**, select **LibraryPrep_RNASeq_ILM_v1.1.rst.**

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

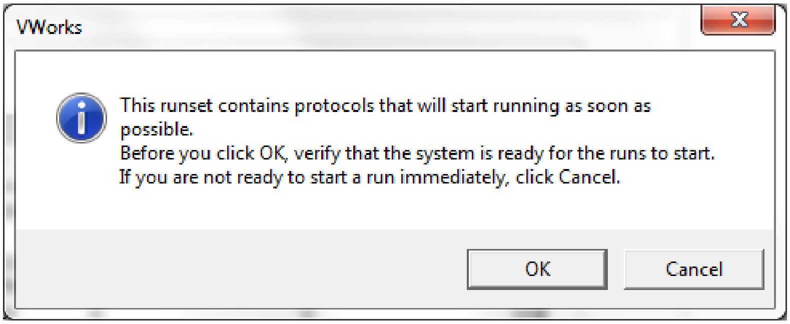
20 Click **Display Initial Workstation Setup**.

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



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

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



This runset contains protocols that will start running as soon as possible.
Before you click OK, verify that the system is ready for the runs to start.
If you are not ready to start a run immediately, click Cancel.

Running the LibraryPrep_RNASeq_ILM_v1.1.rst runset takes approximately 3 hours. Once complete, the purified, adaptor-ligated cDNA samples are located in the Eppendorf plate at position 7 of the Bravo deck.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 4. Index cDNA libraries by PCR amplification

In this step, the Agilent NGS Workstation completes the liquid handling steps for PCR amplification of the adaptor-ligated cDNA samples in a 3-primer PCR that includes the appropriate indexing primer. After the reactions are set up by the workstation, you transfer the PCR plate to a thermal cycler for amplification. The amplification cycle number is based on the initial amount of total RNA sample used for library preparation.

Before you begin, thaw and mix the reagents listed in Table 32 below and keep on ice.

Table 32 Reagents for use in pre-capture PCR protocol

Storage Location	Kit Component	Where Used in Protocol
SureSelect RNA Library Prep, ILM (Pre PCR)[*], –20°C	PCR Master Mix[†] (bottle)	page 67
	Uracil DNA Glycosylase (UDG) (tube with yellow cap)	page 67
	SureSelect Primer (tube with brown cap)	page 67
	ILM Reverse PCR Primer[‡] (tube with black cap)	page 66
	SureSelect^{XT} Indexes, 8 bp (blue 96-well plate)	page 66

[*] May also be labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 1.*

[†] May also be labeled as *RNA Seq PCR Master Mix.*

[‡] May also be labeled as *RNA Seq ILM Reverse PCR Primer.*

Prepare the workstation

1. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
2. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
3. Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.

Prepare the index source plate

4 Add 5 µL of the appropriate indexing primer (Index A01–H06) to the appropriate wells of a PCR plate.

The well position for each index should correspond to the position of the RNA sample assigned to that index in the original total RNA sample plate.

CAUTION

For 96-reaction kits, only use the indexing primers provided in columns 1 through 6 (Indexes A01–H06) in step 4. Provided plates also contain indexing primers in columns 7 through 12, which are used in other SureSelect protocols.

See page 90 for a plate map.

Prepare the PCR reaction mix and the master mix source plate

5 Prepare a 1:20 dilution of the ILM Reverse PCR Primer, according to Table 33.

Table 33 Preparation of reverse primer dilution

Reagent	Volume for up to 96 reactions (includes excess)
Nuclease-free water	114 µL
ILM Reverse PCR Primer	6 µL
Total	**120 µL**

CAUTION

The PCR Master Mix used at this step is highly viscous and thorough mixing prior to use is critical for optimal kit performance. Mix by vortexing at high speed for 5 seconds before removing an aliquot for use and after combining with other solutions as directed in Table 34. Pipetting up and down is not sufficient to mix this reagent.

6 Vortex the thawed vial of PCR Master Mix 5 seconds at high speed to ensure homogeneity.

7 Prepare the appropriate volume of PCR Reaction Mix, according to Table 34. Mix well by vortexing at high speed and keep on ice.

Table 34 Preparation of PCR Reaction Mix

SureSelect 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
PCR Master Mix	25.0 µL	307.5 µL	512.5 µL	717.5 µL	922.5 µL	1332.5 µL	2665 µL
Uracil DNA Glycosylase (UDG)	1.0 µL	12.3 µL	20.5 µL	28.7 µL	36.9 µL	53.3 µL	106.6 µL
SureSelect Primer (Forward primer)	1.0 µL	12.3 µL	20.5 µL	28.7 µL	36.9 µL	53.3 µL	106.6 µL
ILM Reverse PCR Primer (1:20 from Table 33)	1.0 µL	12.3 µL	20.5 µL	28.7 µL	36.9 µL	53.3 µL	106.6 µL
Total Volume	**28 µL**	**344.4 µL**	**574.0 µL**	**803.6 µL**	**1033.2 µL**	**1492.4 µL**	**2984.8 µL**

8 Using the same Nunc DeepWell master mix source plate that was used for the LibraryPrep_RNASeq_ILM_v1.1.rst run, add the volume of PCR Master Mix indicated in Table 35 to all wells of column 6 of the master mix source plate. The final configuration of the master mix source plate is shown in Figure 7.

Table 35 Preparation of the Master Mix Source Plate for TranscriptomePCR_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 Reaction Mix	Column 6 (A6-H6)	39.6 µL	68.3 µL	97.0 µL	125.7 µL	183.1 µL	369.6 µL

NOTE

If you are using a new DeepWell plate for the PCR source plate, leave columns 1 to 5 empty and add the PCR Master Mix to column 6 of the new plate.

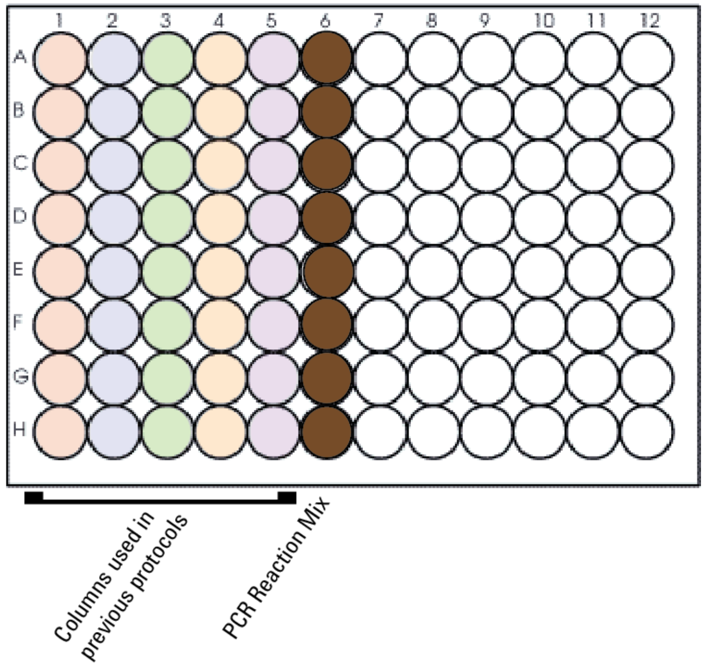


Figure 7 Configuration of the master mix source plate for Transcriptome-PCR_ILM_v1.0.pro. Columns 1-5 were used to dispense master mixes during previous protocols.

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.

NOTE

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

Load the Agilent NGS Workstation

11 Load the Labware MiniHub according to Table 36, using the plate orientations shown in Figure 4.

Table 36 Initial MiniHub configuration for TranscriptomePCR_ILM_v1.0.pro

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty	Empty	Empty	Empty
Shelf 4	Empty	Empty	Empty	Empty
Shelf 3	Empty	Empty	Empty	Empty
Shelf 2	Empty tip box	Empty	Empty	Empty
Shelf 1 (Bottom)	New tip box	Empty	Empty	Empty tip box

12 Load the Bravo deck according to Table 37.

Table 37 Initial Bravo deck configuration for TranscriptomePCR_ILM_v1.0.pro

Location	Content
6	Indexing primers in PCR plate seated on red insert (PCR plate type must be specified on setup form under step 2)
7	Prepped cDNA samples in Eppendorf twin.tec plate, oriented with well A1 in the upper-left
9	Master mix source plate (Nunc DeepWell), unsealed and seated on silver insert

13 Load the BenchCel Microplate Handling Workstation according to Table 38.

Table 38 Initial BenchCel configuration for TranscriptomePCR_ILM_v1.0.pro

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

Run VWorks protocol TranscriptomePCR_ILM_v1.0.pro

14 On the SureSelect setup form, under **Select Protocol to Run**, select **TranscriptomePCR_ILM_v1.0.pro.**

15 Under **Select PCR plate labware for Thermal Cycling**, select the specific type of PCR plate that was loaded on Bravo deck position 6. The plate type selected must be compatible with the thermal cycler to be used for amplification.

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

17 Click **Display Initial Workstation Setup**.

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

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

20 Running the TranscriptomePCR_ILM_v1.0.pro protocol takes approximately 15 minutes. Once complete, the PCR-ready samples, containing prepped DNA, PCR master mix, and indexing primer are located in the PCR plate at position 6 of the Bravo deck and you will see the following prompt:

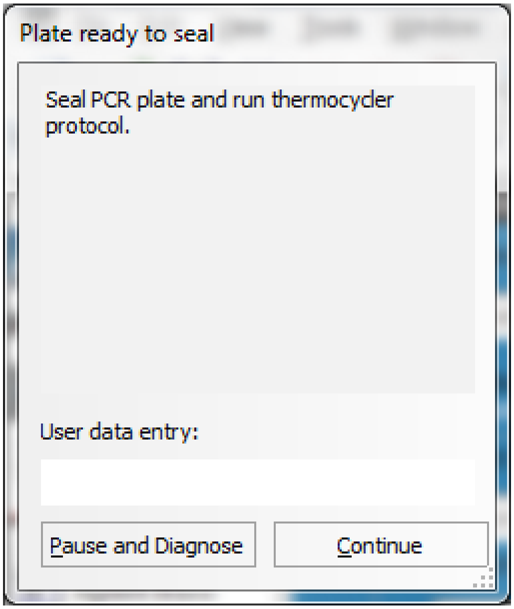


Plate ready to seal

Seal PCR plate and run thermocycler protocol.

- **a** 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.
- **b** Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate air bubbles.
- **c** Transfer the plate to the thermal cycler (with the heated lid ON) and run the program in Table 39.

Table 39 Thermal cycler program for mRNA Library PCR indexing

Segment	Number of Cycles	Temperature	Time
1	1	37°C	15 minutes
2	1	95°C	2 minutes
3	10–16 cycles (see Table 40)	95°C	30 seconds
		65°C	30 seconds
		72°C	1 minute
4	1	72°C	5 minutes
5	1	4°C	Hold

Table 40 mRNA Library PCR indexing cycle number recommendations

Amount of total RNA used for library prep	Cycle Number
50 ng–200 ng	14–16
201 ng–2 µg	12–14
2.1 µg–4 µg	10–12

NOTE

If you started with the minimum total RNA input amount of 50 ng, use 16 amplification cycles in the PCR indexing amplification program (Table 39).

Step 5. Purify amplified DNA using AMPure XP beads

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

Prepare the workstation and reagents

1. Clear the Labware MiniHub and BenchCel of all plates and tip boxes.
2. Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a DNA Away decontamination wipe.
3. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
4. Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
5. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
6. Prepare a Nunc DeepWell source plate for the beads by adding 125 µL of homogeneous AMPure XP beads per well, for each well to be processed.
7. Prepare a Thermo Scientific reservoir containing 30 mL of nuclease-free water.
8. Prepare a separate Thermo Scientific reservoir containing 90 mL of freshly-prepared 70% ethanol.

NOTE The reagents prepared in step 6 to step 8 above will be used for two sequential purification protocols and should be retained on the workstation at the conclusion of this protocol.

9 Load the Labware MiniHub according to Table 41, using the plate orientations shown in Figure 4.

Table 41 Initial MiniHub configuration for AMPureXP_v1.1.pro:Transcriptome PCR

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	Empty	Empty	Empty
Shelf 4	Empty	Empty	Empty	Empty
Shelf 3	Empty	Empty Eppendorf twin.tec Plate	Empty	Empty
Shelf 2	Empty	Nuclease-free water reservoir from step 7	AMPure XP beads in Nunc DeepWell plate from step 6	Empty
Shelf 1 (Bottom)	Empty	70% ethanol reservoir from step 8	Empty	Empty tip box

10 Load the Bravo deck according to Table 42.

Table 42 Initial Bravo deck configuration for AMPureXP_v1.1.pro:Transcriptome PCR

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

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

Table 43 Initial BenchCel configuration for AMPureXP_v1.1.pro:Transcriptome PCR

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

Run VWorks protocol AMPureXP_v1.1.pro:Transcriptome PCR

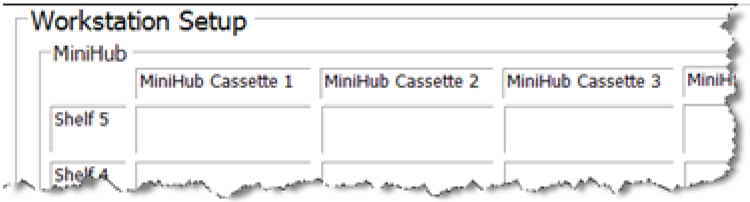
12 On the SureSelect setup form, under **Select Protocol to Run**, select **AMPureXP_v1.1.pro:Transcriptome PCR.**

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

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

15 Click **Display Initial Workstation Setup**.

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



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

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

Step 6. Remove adaptor-dimers using AMPure XP beads

In this step, the Agilent NGS Workstation completes a second round of purification of the amplified cDNA libraries to remove any adaptor-dimers prior to sequencing.

Prepare the workstation and reagents

1. Leave the purification reagents (Nunc plate containing beads and reservoirs containing water and ethanol) in cassettes 2 and 3 of the Labware MiniHub from the previous AMPureXP_v1.1.pro:Transcriptome PCR run. Otherwise, clear the remaining positions of the MiniHub and BenchCel of plates and tip boxes.
2. Leave the waste reservior at position 1 of the Bravo deck. Clear the remaining positions of the Bravo deck of labware.
3. Verify that the ThermoCube at position 9 of the Bravo deck remains set to 0°C.
4. Load the Labware MiniHub according to Table 44, using the plate orientations shown in Figure 4.

Table 44 Initial MiniHub configuration for AMPureXP_v1.1.pro:Transcriptome Dimers

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty Nunc DeepWell plate	Empty	Empty	Empty
Shelf 4	Empty	Empty	Empty	Empty
Shelf 3	Empty	Empty Eppendorf twin.tec Plate	Empty	Empty
Shelf 2	Empty	Nuclease-free water reservoir (retained from previous protocol)	AMPure XP beads in Nunc DeepWell plate (retained from previous protocol)	Empty
Shelf 1 (Bottom)	Empty	70% ethanol reservoir (retained from previous protocol)	Empty	Empty tip box

5 Load the Bravo deck according to Table 45.

Table 45 Initial Bravo deck configuration for AMPureXP_v1.1.pro:Transcriptome Dimers

Location	Content
1	Waste reservoir (Axygen 96 Deep Well Plate, square wells, retained from previous protocol)
9	Once-purified cDNA samples in Eppendorf twin.tec plate seated on red insert (full-skirted twin.tec plate type must be specified on setup form under step 2)

6 Load the BenchCel Microplate Handling Workstation according to Table 46.

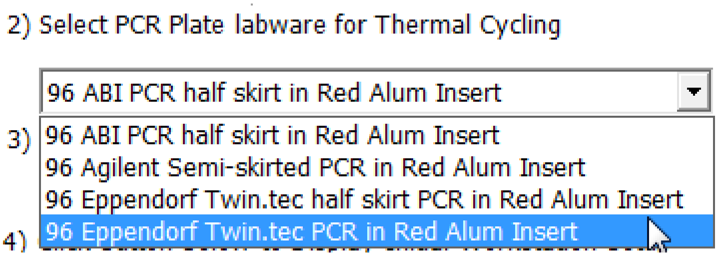
Table 46 Initial BenchCel configuration for AMPureXP_v1.1.pro:Transcriptome Dimers

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

Run VWorks protocol AMPureXP_v1.1.pro:Transcriptome Dimers

7 On the SureSelect setup form, under **Select Protocol to Run**, select **AMPureXP_v1.1.pro:Transcriptome Dimers.**

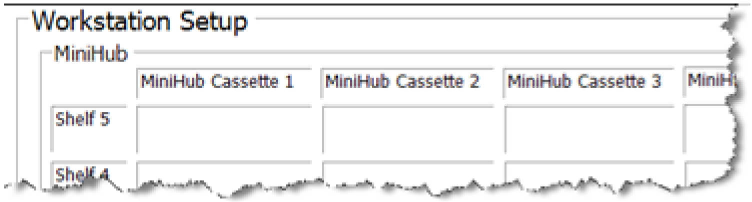
8 Under **Select PCR plate labware for Thermal Cycling**, select **96 Eppendorf Twin.tec PCR in Red Alum Insert**.



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

10 Click **Display Initial Workstation Setup**.

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



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

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

Step 7. Assess Library DNA quantity and quality

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

Use a Bioanalyzer DNA 1000 chip and reagent kit. Perform the assay according to the Agilent DNA 1000 Kit Guide.

1. Set up the 2100 Bioanalyzer as instructed in the reagent kit guide.
2. Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
3. Vortex the plate to mix samples in each well, then centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal.
4. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
5. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
6. Measure the concentration of the library (ng/µL) by integrating under the peak at approximately 200 to 600 bp. For accurate quantification, make sure that the concentration falls within the linear range of the assay. A sample electropherogram is shown in Figure 8.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

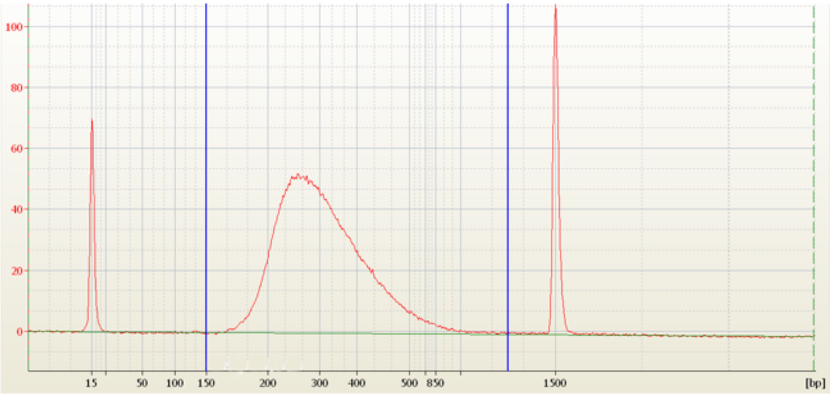


Figure 8 Analysis of amplified library DNA using a DNA 1000 assay.

]

Option 2: Analysis using Agilent 4200 TapeStation and D1000 ScreenTape

Use a D1000 ScreenTape and associated reagent kit to analyze the amplified libraries. For more information to do this step, see the Agilent D1000 Assay Quick Guide.

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

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

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

CAUTION For accurate quantitation, make sure to thoroughly mix the combined DNA and sample buffer by vortexing the assay plate or tube strip for 1 minute on the IKA MS3 vortex mixer provided with the 4200 TapeStation system before loading the samples.

If no IKA MS3 vortex mixer is available, ensure that the samples are mixed thoroughly by vortexing for 10 seconds at high speed on a manual vortex mixer.

4 Load the sample plate or tube strips from step 3, the D1000 ScreenTape, and loading tips into the 4200 TapeStation as instructed in the Assay Quick Guide. Start the run.

5 For each sample, measure the concentration of the library (ng/µL) by integrating under the peak at approximately 200 to 600 bp. A sample electropherogram is shown in Figure 9.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C

.

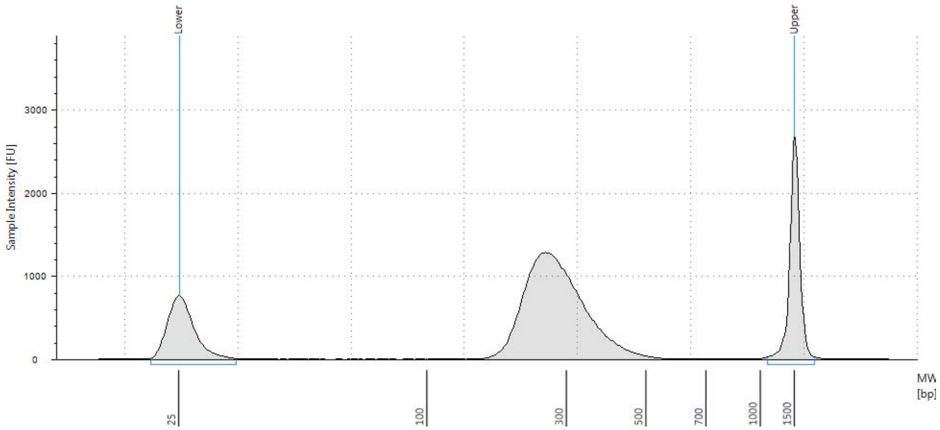


Figure 9 Analysis of amplified library DNA using a D1000 ScreenTape.

Step 8. Pool samples for multiplexed sequencing

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

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 47 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 47 Example of indexed sample volume calculation for total volume of 20 µL

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

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

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

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

Exact library pool dilution and processing can vary based on the flow cell capacity and analysis pipeline versions being used. Refer to the appropriate Illumina user guide for instructions.

Step 9. Prepare and analyze sequencing samples

Proceed to cluster amplification using the Illumina Paired-End Cluster Generation Kit; refer to the manufacturer's instructions for this step. The optimal seeding concentration for cluster amplification from SureSelect mRNA libraries is approximately 10-12 pM.

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

This protocol has been validated with 2 x 100-base paired-end reads. However, read length can be adjusted to achieve the desired research goals.

Sequencing run setup guidelines for 8-bp indexes

Sequencing runs must be set up to perform an 8-nt index read. For the HiSeq platform, use the *Cycles* settings shown in Table 48. 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.

For complete index sequence information, see the Reference chapter starting on page 87.

Table 48 HiSeq platform Run Configuration screen Cycle Number settings

Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	8
Index 2 (i5)	0
Read 2	100

Sequence analysis guidelines

The SureSelect RNA sequencing library preparation method preserves RNA strandedness using dUTP second-strand marking. The sequence of read 1, which starts at the P5 end, matches the reverse complement of the poly-A RNA transcript strand. Read 2, which starts at the P7 end, matches the poly-A RNA transcript strand. When running analysis of this data to determine strandedness, it is important to include this information. For example, when using the Picard tools (https://broadinstitute.github.io/picard) to calculate RNA sequencing metrics, it is important to include the parameter *STRAND_SPECIFICITY= SECOND_READ_TRANSCRIPTION_STRAND* to correctly calculate the strand specificity metrics.

4

Reference

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

Kit Contents

The SureSelect Strand Specific RNA Reagent Kit includes the component kits listed in Table 49. The contents of each component kit are detailed in Table 50 and Table 51.

Table 49 Kit Content

Component Kits	Storage Condition	Part Number
SureSelect RNA Library Prep, ILM (Pre PCR)*	–20°C	5500-0135
SureSelect Poly-A Selection Module (Pre PCR)†	4°C	5190-6411

* May also be labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 1.*

† May also be labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 2.*

Table 50 SureSelect RNA Library Prep, ILM (Pre PCR) Content

Equivalent RNA Library Preparation Kit Component Names[*]		Format (96 Reaction Kit, p/n 5500-0135)
Current name	**Retired name**	
Fragmentation Mix	RNA Seq Fragmentation Mix	bottle
First Strand Master Mix	RNA Seq First Strand Master Mix	tube with orange cap
Second Strand Enzyme Mix[†]	RNA Seq Second-Strand + End Repair Enzyme Mix	bottle
Second Strand Oligo Mix	RNA Seq Second-Strand + End Repair Oligo Mix	tube with yellow cap
dA Tailing Master Mix	RNA Seq dA Tailing Master Mix	bottle
SureSelect Ligation Master Mix	(no change)	tube with purple cap
SureSelect Oligo Adaptor Mix	(no change)	tube with blue cap
PCR Master Mix	RNA Seq PCR Master Mix	bottle
Uracil DNA Glycosylase (UDG)	(no change)	tube with yellow cap
SureSelect Primer	(no change)	tube with brown cap
ILM Reverse PCR Primer	RNA Seq ILM Reverse PCR Primer	tube with black cap
ILM Post-Capture PCR Primer[‡]	RNA Seq ILM Post-Capture PCR Primer	tube with green cap
SureSelect^{XT} Indexes, 8 bp[**]	(no change)	SureSelect 8 bp Indexes A01 through H12, provided in blue 96-well plate[††]

[*] Some component names were updated in June, 2020. Formulations of the reagents supplied and protocols for use of the reagents are unchanged. All components are supported through the expiration date listed on the Certificate of Analysis.

[†] The *Second Strand Enzyme Mix* and *Second Strand Oligo Mix* also supply the reagents used for cDNA end repair; formulations are equivalent to vials labeled as *RNA Seq Second Strand + End Repair Enzyme Mix* and *Second Strand + End Repair Oligo Mix,* respectively.

[‡] The provided ILM Post-capture PCR Primer is not used in the workflow described in this manual.

[**] See Table 53 on page 91 for index sequences.

[††] See Table 52 on page 90 for a plate map. Although the provided plate contains 96 indexing primers, only indexes A01–H06 should be used for the mRNA library preparation workflow. Wells contain enough volume for two mRNA library preparation reactions per index, using the protocol on page 66.

Table 51 SureSelect Poly-A Selection Module (Pre PCR) Content

Kit Component	Format (96 Reaction Kit, p/n 5190-6411)[*]
Oligo(dT) Microparticles	bottle
Bead Binding Buffer	bottle
Bead Washing Buffer	bottle
Bead Elution Buffer	bottle

[*] Component kit part number 5190-6411 labeled as *SureSelect Strand Specific RNA Library Prep, ILM, Box 2* also contains a vial of Nuclease Free Water and contains reagent tubes labeled as *RNA Seq Bead Binding Buffer, RNA Seq Bead Washing Buffer*, and *RNA Seq Bead Elution Buffer*. Formulations of the reagents supplied and protocols for use of these reagents are unchanged. Ordering information for nuclease-free water is provided in Table 1 on page 12.

Table 52 Plate map for SSEL 8bp Indexes A01 through H12 provided in blue plate in Library Prep kit p/n 5500-0135. Use only indexes A01–H06 (Columns 1 to 6) for the mRNA library preparation workflow.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**A01**	**A02**	**A03**	**A04**	**A05**	**A06**	A07	A08	A09	A10	A11	A12
B	**B01**	**B02**	**B03**	**B04**	**B05**	**B06**	B07	B08	B09	B10	B11	B12
C	**C01**	**C02**	**C03**	**C04**	**C05**	**C06**	C07	C08	C09	C10	C11	C12
D	**D01**	**D02**	**D03**	**D04**	**D05**	**D06**	D07	D08	D09	D10	D11	D12
E	**E01**	**E02**	**E03**	**E04**	**E05**	**E06**	E07	E08	E09	E10	E11	E12
F	**F01**	**F02**	**F03**	**F04**	**F05**	**F06**	F07	F08	F09	F10	F11	F12
G	**G01**	**G02**	**G03**	**G04**	**G05**	**G06**	G07	G08	G09	G10	G11	G12
H	**H01**	**H02**	**H03**	**H04**	**H05**	**H06**	H07	H08	H09	H10	H11	H12

Nucleotide Sequences of SureSelect^{XT} Indexes A01 to H12

Each index is 8 nt in length. See page 85 for sequencing run setup requirements for sequencing libraries using 8-bp indexes.

Table 53 SureSelect Indexes, for indexing primers in blue 96-well plates

Index	Sequence	Index	Sequence	Index	Sequence	Index	Sequence
A01	ATGCCTAA	A04	AACTCACC	A07	ACGTATCA	A10	AATGTTGC
B01	GAATCTGA	B04	GCTAACGA	B07	GTCTGTCA	B10	TGAAGAGA
C01	AACGTGAT	C04	CAGATCTG	C07	CTAAGGTC	C10	AGATCGCA
D01	CACTTCGA	D04	ATCCTGTA	D07	CGACACAC	D10	AAGAGATC
E01	GCCAAGAC	E04	CTGTAGCC	E07	CCGTGAGA	E10	CAACCACA
F01	GACTAGTA	F04	GCTCGGTA	F07	GTGTTCTA	F10	TGGAACAA
G01	ATTGGCTC	G04	ACACGACC	G07	CAATGGAA	G10	CCTCTATC
H01	GATGAATC	H04	AGTCACTA	H07	AGCACCTC	H10	ACAGATTC
A02	AGCAGGAA	A05	AACGCTTA	A08	CAGCGTTA	A11	CCAGTTCA
B02	GAGCTGAA	B05	GGAGAACA	B08	TAGGATGA	B11	TGGCTTCA
C02	AAACATCG	C05	CATCAAGT	C08	AGTGGTCA	C11	CGACTGGA
D02	GAGTTAGC	D05	AAGGTACA	D08	ACAGCAGA	D11	CAAGACTA
E02	CGAACTTA	E05	CGCTGATC	E08	CATACCAA	E11	CCTCCTGA
F02	GATAGACA	F05	GGTGCGAA	F08	TATCAGCA	F11	TGGTGGTA
G02	AAGGACAC	G05	CCTAATCC	G08	ATAGCGAC	G11	AACAACCA
H02	GACAGTGC	H05	CTGAGCCA	H08	ACGCTCGA	H11	AATCCGTC
A03	ATCATTCC	A06	AGCCATGC	A09	CTCAATGA	A12	CAAGGAGC
B03	GCCACATA	B06	GTACGCAA	B09	TCCGTCTA	B12	TTCACGCA
C03	ACCACTGT	C06	AGTACAAG	C09	AGGCTAAC	C12	CACCTTAC
D03	CTGGCATA	D06	ACATTGGC	D09	CCATCCTC	D12	AAGACGGA
E03	ACCTCCAA	E06	ATTGAGGA	E09	AGATGTAC	E12	ACACAGAA
F03	GCGAGTAA	F06	GTCGTAGA	F09	TCTTCACA	F12	GAACAGGC
G03	ACTATGCA	G06	AGAGTCAA	G09	CCGAAGTA	G12	AACCGAGA
H03	CGGATTGC	H06	CCGACAAC	H09	CGCATACA	H12	ACAAGCTA

Plasticware quantities for automation protocols

The tables below show the quantity of each plasticware type used in each automation protocol in the workflow. Quantities listed in the tables only include unique labware that was not used in other protocols or runsets. For example, Nunc DeepWell master mix plates may be reused in multiple protocols but are counted below only where first used.

mRNA_Purification_v1.0.pro

Labware	1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Tip boxes (filled)	2	3	4	4	6	10
Empty tip boxes (for waste tips)	2	2	2	2	2	2
Nunc DeepWell Plates	5	5	5	5	5	5
96 Eppendorf twin.tec full-skirt plates	3	3	3	3	3	3
PCR plates (compatible with thermal cycler)	4	4	4	4	4	4
Axygen square-well plate (waste)	1	1	1	1	1	1

AMPureXP_v1.1.pro:First Strand

Labware	1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Tip boxes (filled)	1	1	2	2	3	6
Empty tip boxes (for waste tips)	1	1	1	1	1	1
Nunc DeepWell Plates	2	2	2	2	2	2
96 Eppendorf twin.tec full-skirt plates	1	1	1	1	1	1
Thermo Scientific Reservoirs	2	2	2	2	2	2
Axygen square-well plate (waste)	1	1	1	1	1	1

LibraryPrep_RNASeq_ILM_v1.1.rst

Labware	1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Tip boxes (filled)	2	3	4	5	7	14
Empty tip boxes (for waste tips)	1	1	1	1	1	1
Nunc DeepWell Plates	3	3	3	3	3	3
96 Eppendorf twin.tec full-skirt plates	4	4	4	4	4	4
Thermo Scientific Reservoirs	2	2	2	2	2	2
Axygen square-well plate (waste)	1	1	1	1	1	1

TranscriptomePCR_ILM_v1.0.pro

Labware	1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Tip boxes (filled)	2	2	2	2	2	2
Empty tip boxes (for waste tips)	2	2	2	2	2	2
PCR plates (compatible with thermal cycler)	1	1	1	1	1	1

AMPureXP_v1.1.pro:Transcriptome PCR

Labware	1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Tip boxes (filled)	1	1	2	2	3	6
Empty tip boxes (for waste tips)	1	1	1	1	1	1
Nunc DeepWell Plates	2	2	2	2	2	2
96 Eppendorf twin.tec full-skirt plates	1	1	1	1	1	1
Thermo Scientific Reservoirs	2	2	2	2	2	2
Axygen square-well plate (waste)	1	1	1	1	1	1

AMPureXP_v1.1.pro:Transcriptome Dimers

Labware	1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Tip boxes (filled)	1	1	2	2	3	6
Empty tip boxes (for waste tips)	1	1	1	1	1	1
Nunc DeepWell Plates	1	1	1	1	1	1
96 Eppendorf twin.tec full-skirt plates	1	1	1	1	1	1

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In This Book

This guide contains information to run the SureSelect Automated Strand-Specific RNA Library Prep protocol using the Agilent NGS Workstation.

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