Avida DNA Reagent Kits

Automated using Agilent NGS Workstation Option B

Library preparation, target capture, and indexing for targeted sequencing on the Illumina platform

Protocol

Version C2, November 2025

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

Notices

Manual Part Number

G9409-90010

Edition

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CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

In this Guide...

This guide provides an optimized protocol for preparation of target-enriched Illumina paired-end sequencing libraries using Avida DNA Reagent Kits. Sample processing steps are automated using the Agilent NGS Workstation Option B.

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.

2 Using the Agilent NGS Workstation with the Avida DNA Workflow

This chapter contains an orientation to the Agilent NGS Workstation, an overview of the Avida DNA workflow, and considerations for designing Avida experiments for automated processing using the Agilent NGS Workstation.

3 gDNA Fragmentation

This chapter describes the steps to fragment gDNA samples using mechanical shearing. gDNA samples must be fragmented prior to starting an Avida workflow.

4 Library Preparation and Hybridization

This chapter describes the steps for automated library preparation and hybridization plate setup with the Avida DNA panel. Libraries are prepared using adaptors that include Unique Molecular Identifiers (UMIs).

5 Capture and Washing

This chapter describes the steps for automated capture of targets captured during hybridization with the Avida DNA panel and washing of those targets.

6 Indexing PCR

This chapter describes the steps for automated plate setup for the indexing PCR reactions.

7 Library Purification, Quality Assessment, and Sample Pooling

This chapter describes the steps for automated library purification, quality analysis plate setup, and optional sample pooling.

8 Sequencing and NGS Analysis

This chapter contains guidance on library sequencing and analysis. Refer to your specific Illumina sequencer's user guide for specific instructions on how to perform sequencing.

9 Reference

This section contains reference information, including Reagent Kit contents, index primer pair information, and troubleshooting tips.

What's New in Version C2

- Updated information on Avida panel sizes. See Table 2 on page 13 and the accompanying *Note* below the table.
- Additional sequencing run guidelines, including Illumina Kit Configuration Selection Guidelines, added to "Step 2. Sequence the libraries" on page 80.

What's New in Version C1

- Support for new Avida DNA panels (see Table 2 on page 13 for part numbers).
- Additional information on Avida panel size values and calculations. See Table 2 on page 13, including the accompanying *Note*, for details.
- Correction to the custom AddOn panel size limit provided on page 87.

What's New in Version C0

- Updated throughout to align with the reworked Avida DNA VWorks form (version B1.3.1.2) which now includes automated protocols and runsets for the Avida DNA, Avida Methyl, and Avida Duo Methyl workflows.

What's New in Version B0

- Support for new Avida DNA Discovery Cancer Panel.
- Expanded information on compatible reagent kits for the Agilent Fragment Analyzer system.
- Updated thawing conditions for Adaptor for ILM.
- Expanded recommended Qubit Assay Kits to include Qubit dsDNA HS and BR Assay Kits.
- Updated some of the reagent volumes for preparation of End Prep master mix, Ligation master mix, and Adaptor mix.
- Updated MiniHub configuration for Capture&Wash_Avida protocol.

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.

Make sure you have the most current protocol. Go to www.agilent.com and search for G9409-90010.

NOTE This protocol describes automated Avida DNA sample processing using the Agilent NGS Workstation Option B. For non-automated sample processing procedures see publication G9409-90000.

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

Introduction

Genomic alteration is the underlying cause of many diseases including cancer. Targeted sequencing is widely used to analyze disease related to genomic alterations. There is a growing demand in both research and clinical settings for a simple, fast, and ultra-sensitive targeted sequencing solution to assess various ranges of genomic alterations of disease-related regions particularly with a low DNA input. Avida targeted sequencing is a novel technology developed and perfected to retrieve genomic information from virtually any region of interest even for samples with limited quantity. This assay is particularly optimized for circulating cell free DNA (cfDNA), an emerging analyte demonstrating increasing importance in molecular diagnostics. The technology also supports fragmented genomic DNA (gDNA) isolated from fresh/fresh-frozen samples or from FFPE-derived samples. With a simplified workflow and fast hybridization chemistry, Agilent's Avida targeted sequencing technology can provide in-depth DNA analysis in hours for a broad range of genomic alterations with the highest recovery of input DNA.

Avida Target Enrichment Technology

At the core of the Avida target enrichment technology is an interlocked, three-dimensional structure, designed specifically for synergistic, indirect capture of intended DNA targets. In our proprietary design, a DNA scaffold forms with the intended target molecule when – and only when – more than one bridge probe is hybridized to the same target and stabilized by an anchor probe labeled with biotin (see Figure 1). Compared to traditional hybridization methods that use single and long biotinylated probes, the faster, more efficient Avida hybridization reaction is built on a much shorter, target-specific sequence used in the bridge probes. Consequently, a highly specific capture is achieved when the synergistic hybridization forms this interlocked structure and, in turn, binds to the streptavidin beads.

Avida probe panels are pre-formulated and ready-to-use to deliver consistent and best-in-class performance.

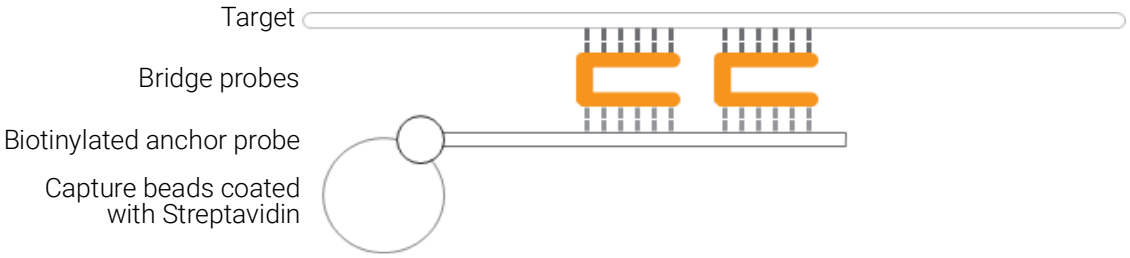


Figure 1 Hybridization of target to biotinylated anchor probe mediated by bridge probes

Procedural and Safety Notes

Important practices for preventing contamination

The Avida DNA technology is highly sensitive, making prevention of contamination of critical importance. Observe the following cautions when performing the workflow.

CAUTION Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe before starting a protocol or runset.

CAUTION 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.

CAUTION To prevent PCR product contamination of samples throughout the workflow:

- Assign separate pre-PCR and post-PCR work areas and use dedicated equipment, supplies, and reagents in each area. In particular, never use materials designated to post-PCR work areas for pre-PCR segments of the workflow. Reagents can be transferred from the pre-PCR area to the post-PCR area, but never from the post-PCR area to the pre-PCR area.
- Maintain clean work areas. Before each assay, clean the surfaces that pose the highest risk of contamination using a solution of 10% bleach and water, or equivalent. Then, wipe the surfaces with water to remove bleach residue.
- Always use dedicated pre-PCR pipettors with nuclease-free aerosol-resistant tips to pipette dedicated pre-PCR solutions.
- Wear powder-free gloves. Use good laboratory hygiene, including changing gloves after contact with any potentially-contaminated surfaces.

Safety notes

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

In general, follow Biosafety Level 1 (BSL 1) safety rules.

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.
- Some protocol 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.
- If your thermal cycler is compatible with the use of compression pads, add a compression pad whenever you load a plate that was sealed with the PlateLoc thermal microplate sealer. The pad improves contact between the plate and the heated lid of the thermal cycler.
- 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 41.
- Reagents requiring –20°C storage are temperature sensitive and appropriate care should be taken during storage and handling. Avoid extensive freeze-thaw cycles. Make sure all reagents previously stored at –20°C are completely thawed before use.
- Possible stopping points, where samples may be stored at 4°C or –20°C, are marked in the protocol.

Materials Provided with Avida DNA Kits and Panels

Table 1 lists the Avida DNA reagent kits and the sub-kit boxes provided with each kit. The 96-reaction kits provide sufficient reagents for 6 assays with 16 samples.

Table 2 lists the Avida DNA panels compatible with this Protocol.

Table 1 Avida DNA reagent kits

Avida DNA reagent kits	Sub-kit boxes	Storage Temperature
P/N G9418A Avida DNA reagent kit with 1-96 index primer pairs for ILM, 96 reactions	Avida DNA Reagent Box 1, 96 Reactions	–20°C
	Avida DNA and Duo Reagent Box 2, 96 Reactions	Room temperature
	Avida Beads Box, 96 Reactions	4°C
	Avida Index Primer Pairs 1–96 for ILM	–20°C
P/N G9418B Avida DNA reagent kit with 97-192 index primer pairs for ILM, 96 reactions	Avida DNA Reagent Box 1, 96 Reactions	–20°C
	Avida DNA and Duo Reagent Box 2, 96 Reactions	Room temperature
	Avida Beads Box, 96 Reactions	4°C
	Avida Index Primer Pairs 97–192 for ILM	–20°C

Table 2 Compatible Avida DNA panels

Capture Probes Panel	Target Size	Probes Size	Design ID	Agilent P/N
Avida DNA Discovery Cancer Panel	1.96Mb	3.43 Mb	D3496941	5280-0045
Avida DNA Expanded Cancer Panel	344 kb	507 kb	D3483241	5280-0048
Avida DNA Focused Cancer Panel	26 kb	41 kb	D3483231	5280-0051
Avida DNA Onco LB Panel	875 kb	1.17 Mb	D3505061	5280-0173
Avida DNA Onco LB Plus Panel	1.75 Mb	2.48 Mb	D3505071	5280-0176
Avida DNA Lymphoma Panel	379 kb	451 kb	D3505051	5280-0179

NOTE

The **target size** is the size of the merged target regions used to design the capture probes for the panel. The **probes size** is the size of the merged genomic regions covered by the capture probes in the panel (see the *Covered.bed* file for the panel). You can download the bed files for a panel from the Agilent SureDesign website.

Certain protocol steps vary based on the Avida DNA panel (i.e., preparing the Hyb master mix on page 38 and programming the thermal cycler for indexing PCR on page 62). At these steps, follow the instructions that pertain to your selected panel.

Online resource for Avida index sequences

Each member of the Avida Index Primer Pairs contains a unique 8-bp P5 or P7 index, resulting in dual-indexed NGS libraries. For complete index sequence information, download the Avida Index Sequence Resource Excel spreadsheet using this link: Avida Index Resource.

Note that this link does not open a website. It automatically downloads the Excel spreadsheet to the default folder for downloaded files saved by your web browser. Locate the file in the folder to open it in Microsoft Excel or another compatible spreadsheet program. You can also find a link to the spreadsheet on the Avida DNA product page on www.agilent.com. The first tab of the spreadsheet provides instructions for use of the spreadsheet contents.

Additional Materials Required

Input DNA samples

The requirements for the DNA samples used as the starting material for the workflow are summarized in Table 3. Input quantity ranges are based on Qubit Assay quantification of the DNA samples. DNA should be stored in low TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) or Qiagen Elution Buffer EB (10 mM Tris-HCl, pH 8.5). DNA needs to be at the appropriate size before you begin library preparation. For gDNA, Agilent recommends using Covaris shearing to achieve average insert sizes of 150–300 base pairs (see Chapter 3, "gDNA Fragmentation" for instructions). Since cfDNA typically has an average size of 170 base pairs, no further fragmentation is required.

Table 3 Input DNA requirements by DNA sample type

Sample type	Input quantity	Fragmentation requirements	Storage buffer
cfDNA	1–100 ng*	No fragmentation required as average fragment size is already 170 bp	Low TE buffer or Qiagen Buffer EB
gDNA from fresh or fresh-frozen sample or FFPE-derived sample	10–100 ng†	Fragmentation with Covaris shearing to insert sizes of 150–300 bp	Low TE buffer or Qiagen Buffer EB

* Libraries can be generated with inputs of cfDNA as low as 1 ng. For variant detection, increasing the input improves performance.

† For FFPE-derived gDNA, use the maximum amount of sample available in this range.

NOTE The Avida workflow is not compatible with the Seraseq ctDNA Complete Reference Material or the Seraseq ctDNA Reference Material V2 available from SeraCare. Contact Agilent Technical Support with questions.

Additional reagents and equipment required

See Table 4 through Table 9 for additional reagents and equipment required to complete the workflow.

Table 4 Additional required reagents

Description	Vendor and Part Number
100% Ethanol (Ethyl Alcohol, 200 proof)	Millipore p/n EX0276, or equivalent
1X Low TE Buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA)	Thermo Fisher Scientific p/n 12090-015, or equivalent
Qubit HS dsDNA Assay Kit (optional) 100 assays 500 assays	Thermo Fisher Scientific p/n Q32851 (HS) or Q32850 (BR) p/n Q32854 (HS) or Q32853 (BR)
Purification Beads: AMPure XP for PCR purification 5 mL 60 mL	Beckman Coulter Genomics p/n A63880 p/n A63881

Table 5 Required equipment

Description	Vendor and Part Number
Thermal cycler and accessories (including compression pads, if compatible)	Various suppliers *Important*: Not all PCR plate types are supported for use in the VWorks automation protocols for the Agilent NGS Workstation. Select a thermal cycler that is compatible with one of the supported PCR plate types. See supported plate types in the listing below.
PCR plates compatible with the Agilent NGS Workstation and associated VWorks automation protocols	Only the following PCR plates are supported: • 96 ABI PCR half-skirted plates (MicroAmp Optical plates), Thermo Fisher Scientific p/n N8010560 • 96 Agilent semi-skirted PCR plate, Agilent p/n 401334 • 96 Eppendorf twin.tec half-skirted PCR plates, Eppendorf p/n 951020303 • 96 Eppendorf twin.tec PCR plates (full-skirted), Eppendorf p/n 951020401
Nucleic acid analysis system (instrument and consumables)	Select one system from Table 8 on page 17
Heat blocks or water baths heated to 37°C and 50°C	General laboratory supplier
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Conical tubes, sterile	Thermo Fisher Scientific p/n 352059 or equivalent
Reagent reservoirs for use with multichannel pipettes	VWR 10015-236 or equivalent
Magnet for 1.5-mL tubes – only required if manually preparing the Capture Beads; see page 50	Thermo Fisher Scientific p/n 12321D or equivalent
Microcentrifuge	Eppendorf microcentrifuge, model 5417C or equivalent
Centrifuge	Eppendorf centrifuge, model 5804 or equivalent
Centrifuge for 96-well plate	General laboratory supplier
Multichannel and single channel pipettes	Rainin Pipet-Lite Multi Pipette or equivalent
Sterile, nuclease-free aerosol barrier pipette tips, vortex mixer, cold rack, ice bucket, and powder-free gloves	General laboratory supplier

Table 6 Bravo NGS Workstation and Workstation plasticware

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 or 13.1.12.1543) OR Agilent p/n G5574AA (VWorks software version 13.1.0.1366 or 13.1.12.1543)
Robotic Pipetting Tips (Sterile, Filtered, 250 µL)	Agilent p/n 19477-022
Processing plates, 96-well, full-skirted	96-well Eppendorf twin.tec plates (Eppendorf p/n 951020401) OR 96-well Armadillo plates (Thermo Fisher Scientific p/n AB2396)
Agilent Reservoirs, Single cavity, 96 pyramids base geometry, 44 mm height – used when workstation setup calls for **Agilent Deep Well Reservoir**	Agilent p/n 201244-100

Table 6 Bravo NGS Workstation and Workstation plasticware (continued)

Description	Vendor and Part Number
Agilent Storage/Reaction Microplates, 96 wells, 1 mL/round well – used when workstation setup calls for **Agilent Deep Well Plate** or **Agilent DW Plate**	Agilent p/n 203426-100
Agilent Storage/Reaction Microplates, 96 wells, 2 mL/square well – used when workstation setup calls for **Waste Plate (Agilent 2 mL Square Well)**	Agilent p/n 201240-100

Table 7 Recommended supplies for decontaminating the NGS Workstation surfaces

Description	Vendor
Dilute bleach (10%) wipes	Hype-Wipe Bleach Towelettes, or equivalent
Isopropanol (70%) wipes	VWR Pre-Moistened Clean Wipes, or equivalent

Table 8 Nucleic acid analysis platform options -- select one

Analysis System	Vendor and Part Number Information
Agilent 4200/4150 TapeStation Instrument	Agilent p/n G2991AA/G2992AA
Consumables:	
96-well sample plates	p/n 5042-8502
96-well plate foil seals	p/n 5067-5154
8-well tube strips	p/n 401428
8-well tube strip caps	p/n 401425
D1000 ScreenTape	p/n 5067-5582
D1000 Reagents	p/n 5067-5583
Agilent 5200/5300/5400 Fragment Analyzer Instrument	Agilent p/n M5310AA/M5311AA/M5312AA
Consumables:	
Select from the following kits[*]	
NGS Fragment Kit (1-6000 bp)	p/n DNF-473-0500
Small Fragment Kit (1-1500 bp)	p/n DNF-476-0500
HS NGS Fragment Kit (1-6000 bp)	p/n DNF-474-0500
HS Small Fragment Kit (1-1500 bp)	p/n DNF-477-0500

* Consider factors such as sample type and quality, expected target insert size, and sample input quantity when selecting a DNA Analysis Kit for the Fragment Analyzer system.

Equipment required for gDNA fragmentation

Table 9 lists information on equipment for fragmentation of gDNA samples using mechanical shearing. Sample fragmentation is required for gDNA samples but not for cfDNA samples.

Table 9 Equipment for mechanical shearing of gDNA samples

Description	Vendor and Part Number	Usage Notes
Covaris Sample Preparation System	Covaris model E220	Additional Covaris instrument models and sample holders may be used after optimization of shearing conditions.
Covaris microTUBE sample holders	Covaris p/n 520045	

2 Using the Agilent NGS Workstation with the Avida DNA Workflow

This chapter contains an orientation to the Agilent NGS Workstation, an overview of the Avida DNA workflow, and considerations for designing Avida 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 10.

Review the user guides listed in Table 10 (available at www.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 specifically for the Avida DNA workflow are detailed in this user guide.

Table 10 Agilent NGS Workstation components User Guide reference information

Device	User Guide part number
Bravo Platform	SD-V1000376 (formerly G5562-90000)
VWorks Software	G5415-90068
BenchCel Microplate Handler	G5580-90000
Labware MiniHub	G5584-90001
PlateLoc Thermal Microplate Sealer	G5585-90010

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 three interchangeable disposable-tip pipette heads, it accurately dispenses fluids from 0.3 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 2 to familiarize yourself with the location numbering convention on the Bravo platform deck.

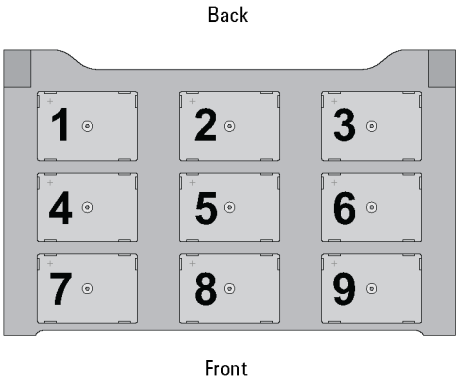


Figure 2 Bravo platform deck

Setting the Temperature of Bravo Deck Heat Blocks

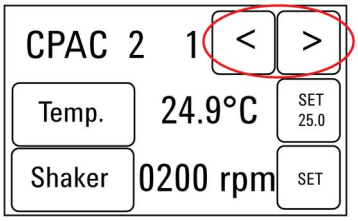
Bravo deck positions 4 and 6 are equipped with Inheco heat blocks, used to incubate sample plates at defined temperatures during the run. Runs that include high- (85°C) or low- (4°C) temperature incubation steps may be expedited by pre-setting the temperature of the affected block before starting the 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 11 for designations of the heat block-containing Bravo deck positions on the Multi TEC control device.

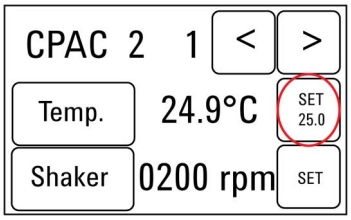
Table 11 Inheco Multi TEC Control touchscreen designations

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

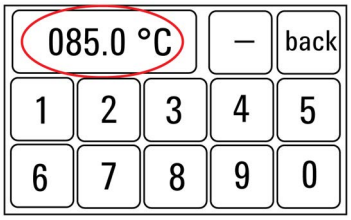
1 Using the arrow buttons, select the appropriate block (CPAC 2 block 1 or CPAC 2 block 2).



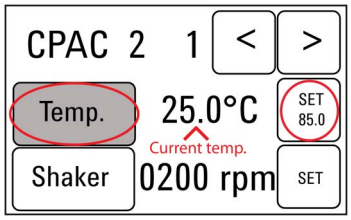
2 To set the temperature of the selected block, press the SET button.



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



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



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

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

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

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

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

3 Press the START button.

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

VWorks Automation Control Software

VWorks software, included with your Agilent NGS 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 system liquid handling protocols for the Avida DNA workflow. General instructions for starting up the VWorks software and the included protocols are provided below. Each time a specific VWorks protocol is used in the Avida DNA 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 versions 13.1.0.1366 and 13.1.12.1543.

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

Logging in to the VWorks software

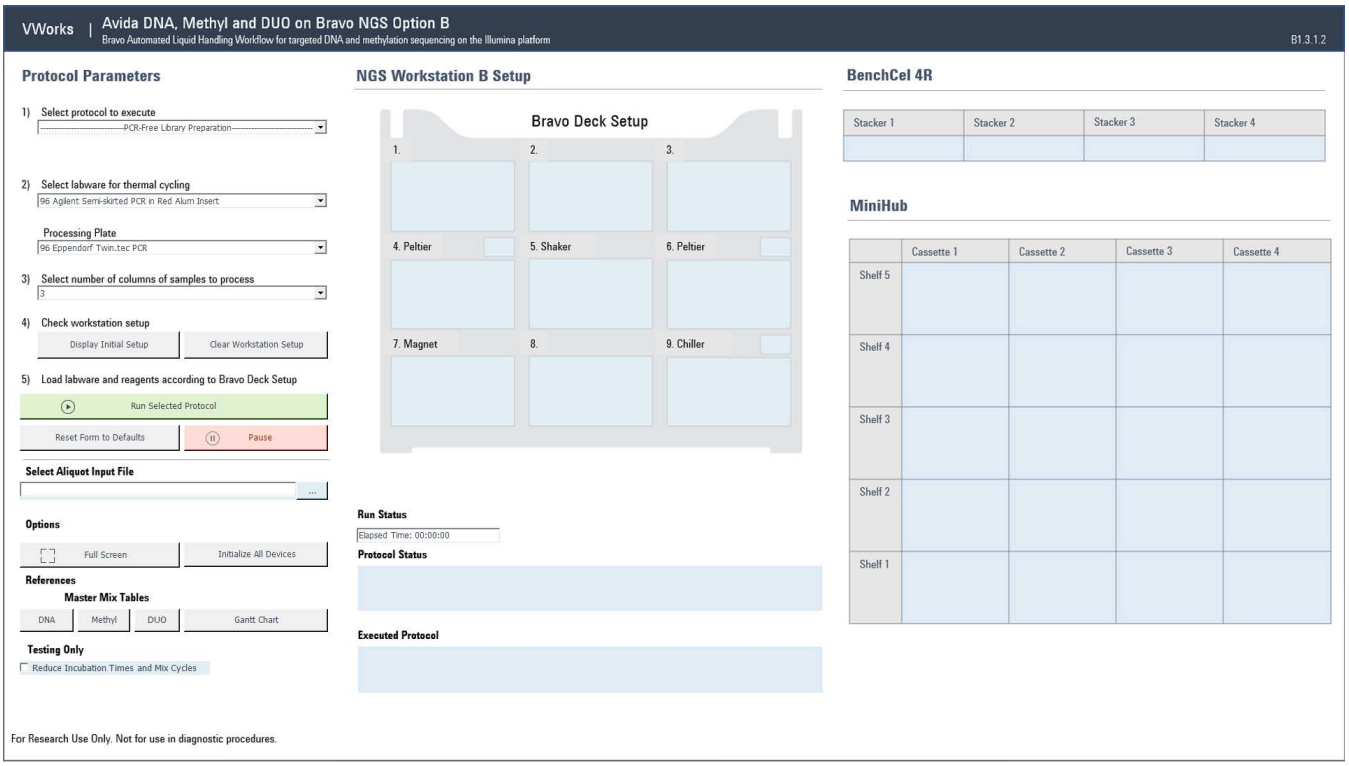
1. Double-click the VWorks icon for the Avida DNA VWorks Form shortcut on the Windows desktop to start the VWorks software.
2. If User Authentication dialog is not visible, click **Log in** on the VWorks window toolbar.
3. In the User Authentication dialog, type your VWorks user name and password, and 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 Avida Form to setup and start a run

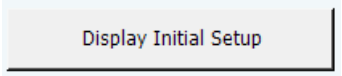
Use the Avida VWorks form, shown below, to set up and start each automation protocol or runset.



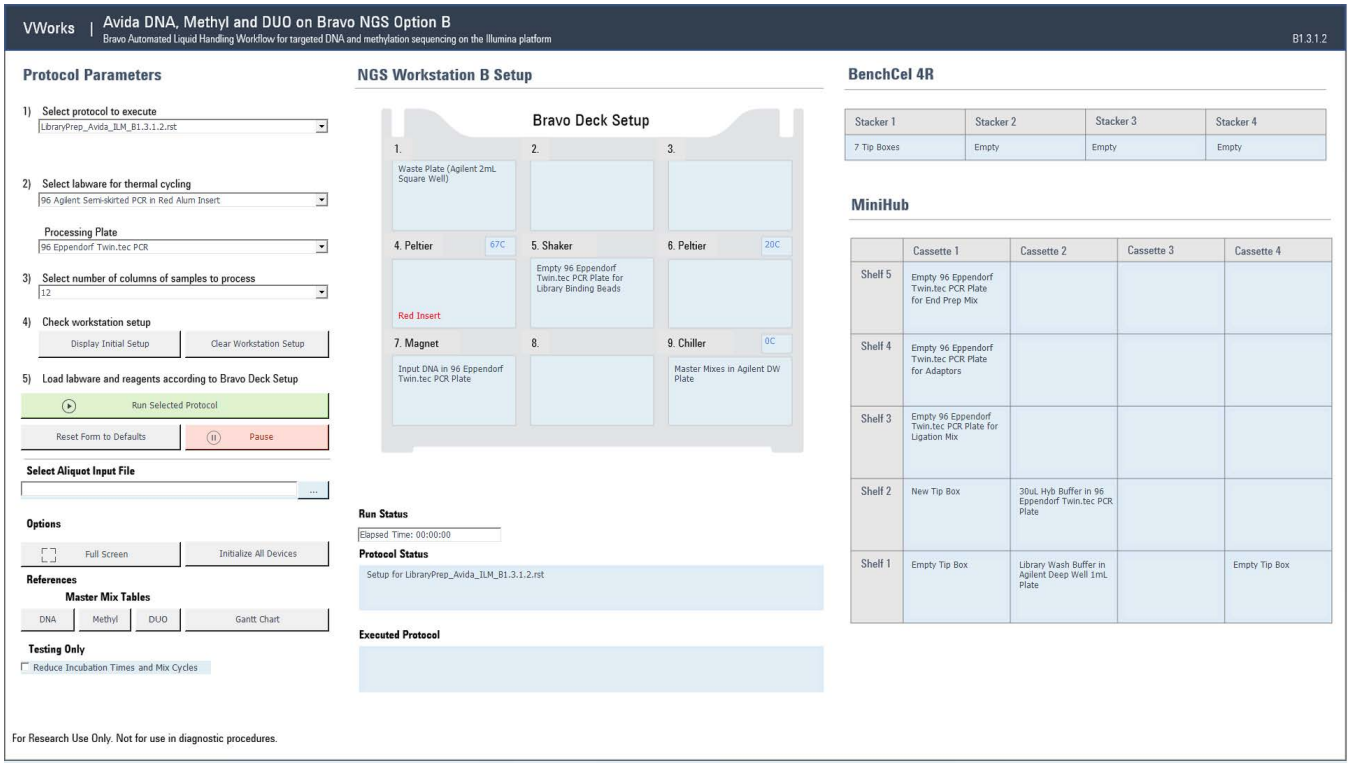
1 Open the form using the shortcut (shown below) on your desktop, or by opening the file **Avida_DNA_B1.3.x.x.VWForm** in the directory **C:\VWorks Workspace\NGS Option B\ Avida_DNA_B1.3.x.x\Forms** (where x.x is the version number).

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

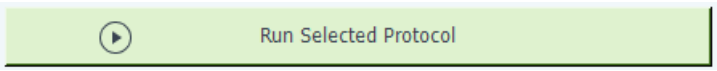
3 Once all run parameters have been specified on the form, click **Display Initial Setup**.



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



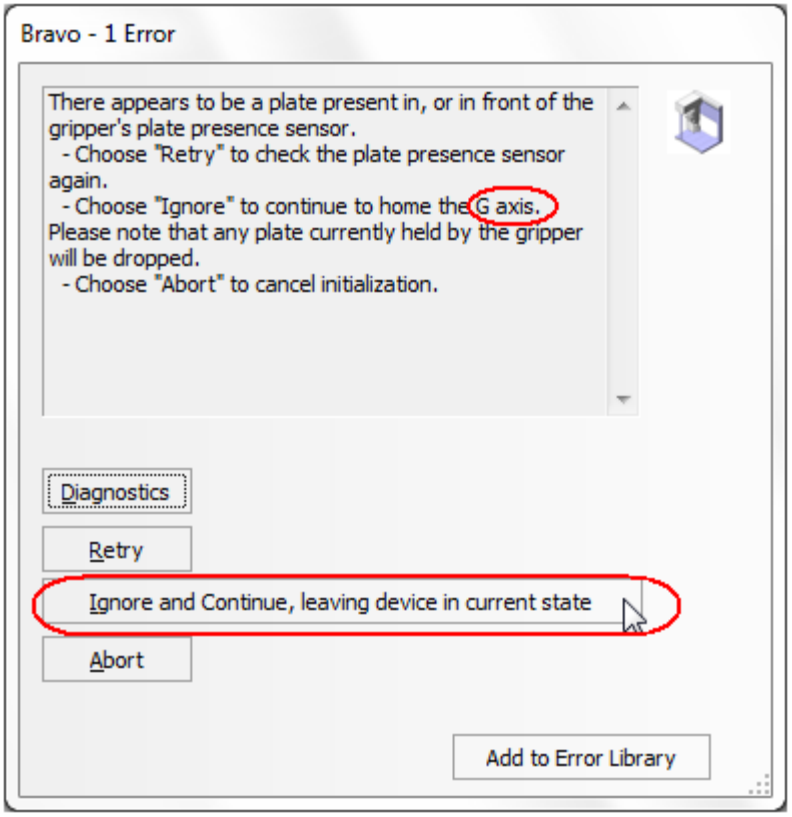
5 After verifying that the NGS Workstation has been set up correctly, click **Run Selected Protocol**.



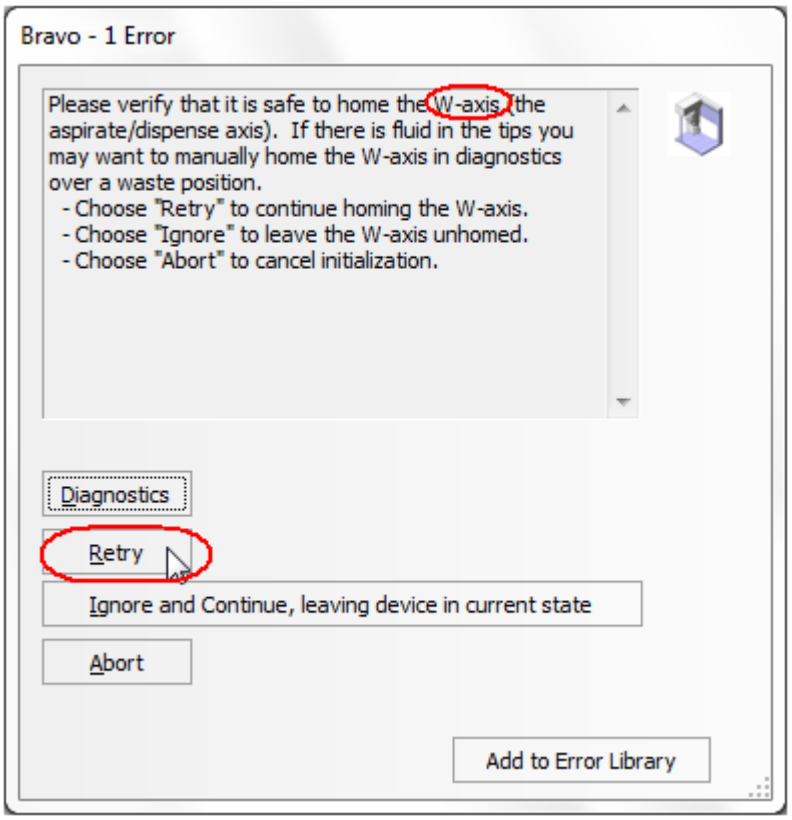
Error messages encountered at start of run

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

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



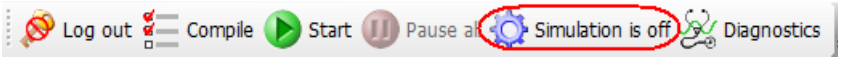
2 If you encounter the W-axis error message shown below, select **Retry**.



Verifying the Simulation setting

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

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



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

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

Automation Protocols for the Avida DNA Workflow

The starting material for the Avida DNA workflow is cell-free DNA (cfDNA) or fragmented genomic DNA (gDNA) isolated from fresh/fresh-frozen samples or from FFPE-derived samples (refer to "Input DNA samples" on page 15 for detailed requirements on the DNA starting material).

The workflow features a PCR-free library preparation with a proprietary pre-amplification target capture process that maximizes capture efficiency, specificity, and uniformity. This streamlined protocol delivers high conversion and library complexity which enables highly sensitive detection of genomic changes.

The VWorks form for Avida includes protocols for all three Avida workflows – Avida DNA, Avida Methyl, and Avida Duo Methyl. Table 12 summarizes the VWorks protocols and runsets used during the Avida DNA workflow.

Table 12 Avida DNA workflow automated protocols/runsets

VWorks Protocol/Runset	Description	Approximate Time (12-column run)
LibraryPrep_Avida_ILM	Prepares duplex, molecular-barcoded DNA libraries. Sets up hybridization reactions with the Avida DNA panel to be run on the thermal cycler.	3 hours (+ 1 hour for hybridization)
PrepareCaptureBeads	Prepares the Capture Beads needed for the Target Capture runset.	20 minutes
TargetCaptureDNA	Captures targets from the hybridization and washes the captured DNA hybrids.	50 minutes
PCR_Avida_ILM	Amplifies libraries to incorporate sample indexes and P5/P7 sequences.	15 minutes (+ 0.5–1 hour for PCR)
AMPureXP_Cleanup	Performs purification of sequencing libraries.	35 minutes
TS_D1000	Sets up plate for quality assessment of final libraries on the Agilent TapeStation platform.	10 minutes (+ 1.5 hours on TapeStation)
Aliquot_Captures	Pools indexed DNA libraries.	up to 1 hour

Experimental Setup Considerations for Automated Runs

Considerations for Experimental Setup

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

Table 13 Columns to Samples Equivalency

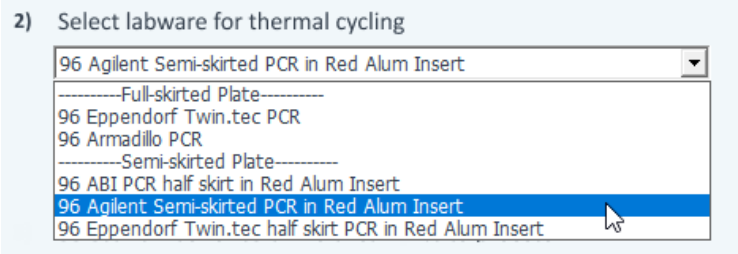
Number of Columns Processed	Total Number of Samples Processed
1	8
2	16
3	24
4	32
6	48
12	96

- The number of columns or samples that may be processed using the supplied reagents (see Table 1) will depend on the experimental design. For greatest efficiency of reagent use, plan experiments using at least 3 columns per run. Each 96-reaction kit contains sufficient reagents for 96 reactions configured as 4 runs of 3 columns of samples per run.
- The Agilent NGS Workstation processes samples column-wise beginning at column 1. The DNA 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.

Plate Type Considerations

PCR plate types

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 on the VWorks Form which PCR plate type will be used to allow correct configuration of the liquid handling components. 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 14.



CAUTION

The plates listed in Table 14 are compatible with the Agilent NGS Bravo and associated VWorks automation protocols, designed to support use of various thermal cyclers.

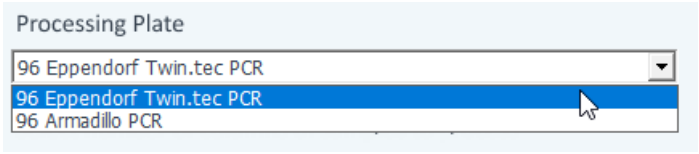
Do not use PCR plates that are not listed in Table 14, even if they are compatible with your chosen thermal cycler.

Table 14 Ordering information for supported PCR plates

Description in VWorks menu	Vendor and part number
-----Full-skirted Plate-----	
96 Eppendorf Twin.tec PCR	Eppendorf p/n 951020401
96 Armadillo PCR	Thermo Fisher Scientific p/n AB2396
-----Semi-skirted Plate-----	
96 ABI PCR half-skirt (MicroAmp Optical plates)	Thermo Fisher Scientific p/n N8010560
96 Agilent Semi-skirted PCR	Agilent p/n 401334
96 Eppendorf Twin.tec half-skirt PCR	Eppendorf p/n 951020303

Processing plate types

Many of the automation protocols use a 96-well processing plate for reagent storage and mixing. The Avida VWorks form allows you to select between two different processing plates, as shown in the menu below. Before running a protocol, verify that the correct processing plate is selected in the menu. Use the same type of processing plate for all protocols and runsets in the same run.



The processing plate is either a 96-well Eppendorf twin.tec PCR plate (Eppendorf p/n 951020401) or a 96-well Armadillo PCR plate (Thermo Fisher Scientific p/n AB2396). Do not substitute another plate type.

3
gDNA Fragmentation

This chapter describes the steps to fragment gDNA samples using mechanical shearing. gDNA samples must be fragmented prior to starting an Avida workflow.

Fragmentation is only required for gDNA samples. If you are using cfDNA samples, proceed to Chapter 4, “Library Preparation and Hybridization.”

NOTE All liquid handling steps in this chapter are performed manually (no automation protocols).

Step 1. Prepare for shearing

NOTE The shearing instructions provided here were optimized using a Covaris model E220 instrument and the 130-µl Covaris microTUBE to generate fragment sizes of 150–300 bp. Consult the manufacturer's recommendations for use of other Covaris instruments or sample holders to achieve the same target gDNA fragment size.

1. Set up the Covaris instrument according to the manufacturer's instructions. Allow enough time (typically 30–60 minutes) for instrument degassing and water bath chilling before starting the protocol.
2. Dilute 10–100 ng of each gDNA sample with 1X low TE buffer to a final volume of 50 µL. Vortex well to mix, then spin briefly. Keep the samples on ice.

Step 2. Shear the gDNA samples

1. For each gDNA sample, transfer the 50-µL sample into a Covaris microTUBE.
2. Spin the microTUBE for 30 seconds to collect the liquid and to remove any bubbles from the bottom of the tube.
3. Secure the microTUBE in the tube holder and shear the gDNA with the settings in Table 15.

Table 15 Shear settings for Covaris E-series instrument (SonoLab software v7 or later)

Setting	High-quality gDNA	FFPE-derived gDNA
Duty Factor	10%	10%
Peak Incident Power (PIP)	175	175
Cycler per Burst	200	200
Treatment Time	2 × 60 seconds (see two-round instructions below)	240 seconds
Bath Temperature	2° to 8°C	2° to 8°C

Use the steps below for two-round shearing of high-quality gDNA samples only:

- **a** Shear for 60 seconds
- **b** Spin the microTUBE for 10 seconds
- **c** Vortex the microTUBE at high speed for 5 seconds
- **d** Spin the microTUBE for 10 seconds
- **e** Repeat step a through step d

4. After shearing, put the Covaris microTUBE back into the loading and unloading station.
5. Spin the microTUBE briefly. While keeping the snap-cap on, insert a pipette tip through the pre-split septum, then slowly remove the sheared gDNA.
6. Transfer the sheared gDNA sample (approximately 50 µL) to a 96-well plate well. Keep the samples on ice.

7 After transferring the gDNA sample, spin the microTUBE briefly to collect any residual sample volume. Transfer any additional collected liquid to the sample well used in step 6 above.

The 50-µL sheared gDNA samples are now ready for library preparation. Proceed directly to Chapter 4, "Library Preparation and Hybridization."

This is not a stopping point.

4 Library Preparation and Hybridization

This chapter describes the steps for automated library preparation and hybridization plate setup with the Avida DNA panel. Libraries are prepared using adaptors that include Unique Molecular Identifiers (UMIs).

The Library Prep automation runset (LibraryPrep_Avida_ILM) directs the NGS Workstation to complete the steps for end-repair, dA-tailing, and ligation of adaptors. Then, the workstation prepares the plate of hybridization reactions for transfer to the thermal cycler.

This chapter uses the reagents listed in Table 16.

Table 16 Reagents for PCR-free library preparation and hybridization

Reagent	Usage Notes	Kit/Storage Location
End Prep Buffer (tube with purple cap)	Thaw at room temperature; vortex before use.	Avida DNA Reagent Box 1, stored at –20°C
End Prep Enzyme (tube with blue cap)	Place on ice or cold block just before use.	
Ligation Buffer (bottle)	Thaw at room temperature; vortex before use.	
Ligation Enzyme (tube with yellow cap)	Place on ice or cold block just before use.	
Adaptor for ILM (tube with orange cap)	Thaw at room temperature then keep on ice.	
Hyb Blocker (tube with red cap)	Thaw at room temperature.	
Hyb Buffer (bottle)	Heat to 37°C for at least 20 minutes before use. Once solution clears, keep at room temperature.	
Hyb Enhancer (amber tube with green cap)	Thaw at room temperature. Thoroughly vortex to solubilize any precipitates.	
Library Binding Beads (bottle)	Equilibrate to room temperature for at least 15 minutes; vortex before use. Some bead aggregates may remain even after vortexing.	Avida Beads Box, stored at 4°C
Nuclease-Free Water (bottle)	—	Avida DNA and Duo Reagent Box 2, stored at Room Temperature
Library Wash Buffer (bottle)	—	
Avida DNA Panel	Thaw at room temperature then keep on ice.	Stored at –20°C

NOTE

Prior to starting a workflow, begin preheating the stock bottle of Hyb Wash Buffer 1 to ensure that it is sufficiently heated and solubilized prior to its use in Chapter 5. The Hyb Wash Buffer 1 is provided in the Avida DNA and Duo Reagent Box 2.

- If a water bath is available, heat the stock bottle in a 50°C water bath for 10 minutes. Then, transfer the bottle to a heat block set to 50°C. Leave the bottle on the 50°C heat block until use.
- If a water bath is not available, place the stock bottle on a heat block set to 50°C. Leave the bottle on the 50°C heat block until use.

If the bottle does not fit in the heat block, place the bottle on top of the heat block, setting it on its side to increase surface area contact. A thermal cycler thermal block set to 50°C may be used in place of a heat block.

Step 1. Prepare the NGS Workstation

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 decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 67°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Pre-set the temperature of Bravo deck position 6 to 20°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.
5. 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.

Step 2. Prepare plates for the DNA samples, Hyb Buffer, and Library Wash Buffer

Automated runs may include 1, 2, 3, 4, 6, or 12 columns of DNA samples to be enriched for sequencing on the Illumina platform. Plan your experiments using complete columns of samples.

Prepare the DNA sample plate (processing plate)

NOTE

On the VWorks form, if you plan to select one of the full-skirted plates for thermal cycling (Eppendorf twin.tec or Armadillo), make sure to use that same plate type for preparation of the DNA sample plate. The NGS Workstation uses the DNA sample plate as the thermal cycler plate.

If you select one of the semi-skirted plates for thermal cycling, then the NGS Workstation transfers the DNA samples to that plate at the end of the runset.

1 In the wells of a processing plate (Eppendorf twin.tec or Armadillo plate), add an appropriate quantity of DNA sample (based on sample type, see below) in a volume of 50 µL. If the sample volume is <50 µL, add Nuclease-Free Water to bring the volume to 50 µL. See Table 3 on page 15 for complete information on the DNA sample input requirements.
   - cfDNA samples: 1–100 ng
   - fragmented gDNA samples: 10–100 ng

NOTE Load the DNA samples into the plate 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.

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

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

Prepare the Hyb Buffer plate (processing plate)

1 Heat the stock bottle of Hyb Buffer to 37°C in a water bath or heat block for at least 20 minutes, or until the Hyb Buffer is completely solubilized. If the Hyb Buffer bottle does not fit into the heat block, place the bottle on its side (to increase surface area contact) on top of the heat block.

   If your laboratory does not have a water bath or heat block, you can heat the bottle of Hyb Buffer by placing it on top of the thermal block of a thermal cycler set to 37°C. More time may be required to completely solubilize the buffer with this technique.

2 Add the required volume of Hyb Buffer to the appropriate wells of a processing plate (Eppendorf twin.tec or Armadillo) based on the Avida DNA panel.
   - 30 µL/well - For Avida DNA Focused Cancer Panel, Expanded Cancer Panel, and Lymphoma Panel
   - 31 µL/well - For Avida DNA Discovery Cancer Panel, Onco LB Panel, and Onco LB Plus Panel

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

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

5 Keep the stock bottle of Hyb Buffer at room temperature until needed again in "Capture Beads Preparation" on page 47.

Prepare the Library Wash Buffer plate (Agilent Deep Well plate)

1 In the wells of an Agilent Deep Well plate, add 400 µL of Library Wash Buffer to each well of each column included in the run.

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

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

Step 3. Prepare the End Prep master mix

1. Thaw the End Prep Buffer then vortex to mix. Inspect the buffer to make sure that no small particles are visible. If particles are visible, continue to vortex until they dissolve.
2. Prepare the appropriate volume of End Prep master mix at room temperature using volumes listed in Table 17. Gently vortex the End Prep master mix at low speed, then briefly spin. Once prepared, keep the End Prep master mix on ice.

Table 17 Preparation of End Prep master mix in a 1.5-mL tube

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
End Prep Buffer (purple cap)	7 µL	98 µL	154 µL	204 µL	266 µL	406 µL	806 µL
End Prep Enzyme (blue cap)	3 µL	42 µL	66 µL	87 µL	114 µL	174 µL	346 µL
Total Volume	**10 µL**	**140 µL**	**220 µL**	**291 µL**	**380 µL**	**580 µL**	**1152 µL**

Step 4. Prepare the Ligation master mix

1. Thaw the Ligation Buffer at room temperature then vortex to mix. Inspect the buffer to make sure that no small particles are visible. If particles are visible, continue to vortex until they dissolve.
2. Prepare the appropriate volume of Ligation master mix at room temperature using volumes listed in Table 18. Vortex the Ligation master mix, then briefly spin. Once prepared, keep the Ligation master mix on ice.

Table 18 Preparation of Ligation master mix in a 1.5-mL tube or conical tube

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 (bottle)	16 µL	166 µL	314 µL	456 µL	602 µL	920 µL	1800 µL
Ligation Buffer (bottle)	25 µL	260 µL	490 µL	712 µL	942 µL	1436 µL	2814 µL
Ligation Enzyme (yellow cap)	6 µL	62 µL	118 µL	172 µL	226 µL	344 µL	676 µL
Total Volume	**47 µL**	**488 µL**	**922 µL**	**1340 µL**	**1770 µL**	**2700 µL**	**5290 µL**

Step 5. Prepare the Adaptor mix

1. Thaw the Adaptor for ILM at room temperature then vortex to mix. Keep the thawed Adaptor for ILM on ice.
2. Prepare the appropriate volume of Adaptor mix on ice using the volumes listed in Table 19. Vortex the Adaptor mix, then briefly spin. Keep the Adaptor mix on ice.

Table 19 Preparation of Adaptor mix in a 1.5-mL tube

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 (bottle)	2.5 µL	36 µL	54 µL	74 µL	96 µL	146 µL	286 µL
Adaptor for ILM (orange cap)	5 µL	73 µL	107 µL	147 µL	193 µL	293 µL	573 µL
Total Volume	**7.5 µL**	**109 µL**	**161 µL**	**221 µL**	**289 µL**	**439 µL**	**859 µL**

Step 6. Prepare the Hyb master mix

1 Thaw the Hyb Blocker, Hyb Enhancer, and Avida DNA Panel at room temperature. Transfer the Avida DNA Panel to ice once thawed.

2 Prepare the Hyb master mix using the volumes in either Table 20 or Table 21. Select the appropriate table based on the Avida DNA panel. Vortex the Hyb master mix, then briefly spin. Keep the Hyb master mix at room temperature.

Use Table 20 for the following Avida DNA panels.

- Avida DNA Expanded Panel, Focused Panel, and Lymphoma Panel

Use Table 21 for the following Avida DNA panels.

- Avida DNA Discovery Panel, Onco LB Panel, and Onco LB Plus Panel

NOTE To include a custom Avida AddOn panel in the Hyb master mix, see the protocol adjustments described in "Avida Custom AddOn Panels" on page 87. Custom Avida AddOn panels can be created on the SureDesign website.

Table 20 Preparation of Hyb master mix in a 1.5-mL tube or conical tube – for Expanded, Focused, Lymphoma

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 (bottle)	17.5 µL	222 µL	390 µL	554 µL	718 µL	1050 µL	2036 µL
Hyb Blocker (red cap)	2.5 µL	32 µL	56 µL	80 µL	102 µL	150 µL	290 µL
Hyb Enhancer (amber tube with green cap)	6 µL	76 µL	134 µL	190 µL	246 µL	360 µL	698 µL
Avida DNA panel	4 µL	50 µL	90 µL	126 µL	164 µL	240 µL	466 µL
Total Volume	**30 µL**	**380 µL**	**670 µL**	**950 µL**	**1230 µL**	**1800 µL**	**3490 µL**

Table 21 Preparation of Hyb master mix in a 1.5-mL tube or conical tube – for Discovery, Onco LB, Onco LB Plus

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 (bottle)	17.5 µL	224 µL	392 µL	556 µL	724 µL	1056 µL	2046 µL
Hyb Blocker (red cap)	2.5 µL	32 µL	56 µL	80 µL	104 µL	150 µL	292 µL
Hyb Enhancer (amber tube with green cap)	5 µL	64 µL	112 µL	158 µL	206 µL	302 µL	584 µL
Avida DNA panel	4 µL	52 µL	90 µL	126 µL	166 µL	242 µL	468 µL
Total Volume	**29 µL**	**370 µL**	**650 µL**	**920 µL**	**1200 µL**	**1750 µL**	**3390 µL**

Step 7. Prepare the Library Prep master mix reagent source plate

The Library Prep master mix reagent source plate is an **Agilent Deep Well plate** containing the Avida reagents used in the Library Prep runset.

1 Using an Agilent Deep Well plate, prepare the master mix reagent source plate for the Library Prep runset by adding the required reagents to the appropriate wells. Keep the plate on ice during and after preparation.

Table 22 lists the required volumes and well positions for each reagent based on the number of columns in the run. Figure 3 shows the configuration of the reagent source plate.

Table 22 Volumes (per well) for the Library Prep master mix reagent source plate based on number of columns in the run

Master Mix Solution	Position on Source Plate	Volume of master mix added per well of source plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Library Binding Beads	Column 1 (A1-H1)	100 µL	190 µL	280 µL	370 µL	550 µL	1090 µL
End Prep master mix	Column 2 (A2-H2)	17 µL	27 µL	36 µL	47 µL	71 µL	139 µL
Adaptor mix	Column 3 (A3-H3)	13 µL	20 µL	27 µL	36 µL	54 µL	106 µL
Ligation master mix	Column 4 (A4-H4)	58 µL	109 µL	160 µL	211 µL	318 µL	624 µL
Hyb master mix	Column 5 (A5-H5)	46 µL	80 µL	114 µL	148 µL	216 µL	420 µL

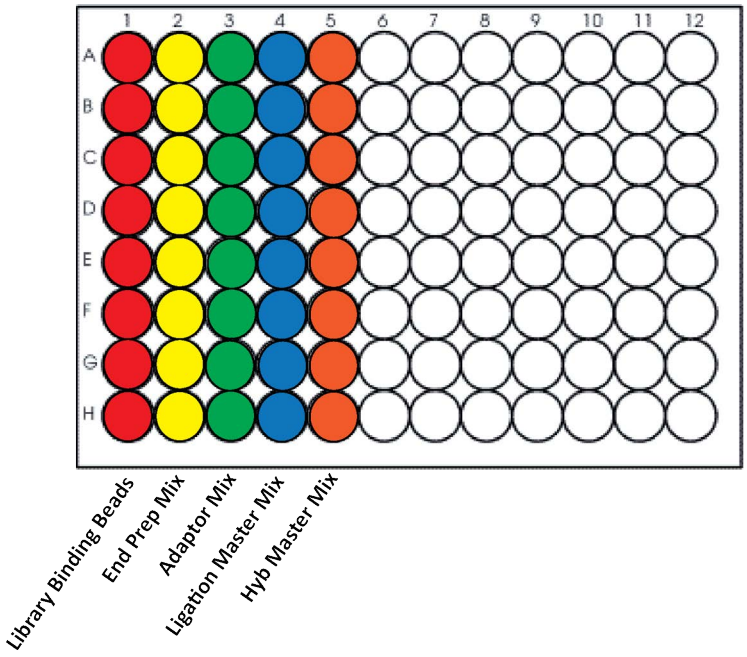


Figure 3 Configuration of the Library Prep master mix source plate for runset LibraryPrep_Avida_ILM

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

3 Centrifuge the plate at 50 × g for 1 second to drive the well contents off the walls and plate seal and to eliminate any bubbles. Keep the 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.

Step 8. Load the NGS Workstation

1 Using the plate orientations shown in Figure 4, load the Labware MiniHub according to Table 23.

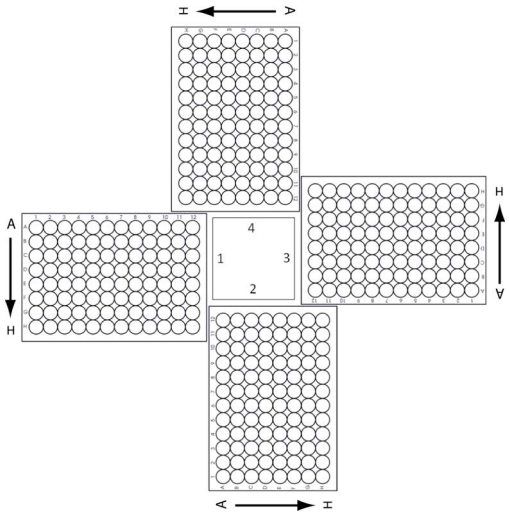


Figure 4 Agilent Labware MiniHub plate orientation.

Table 23 Initial MiniHub configuration for LibraryPrep_Avida_ILM runset

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty processing plate (Eppendorf twin.tec or Armadillo) – used for reagent processing	—	—	—
Shelf 4	Empty processing plate (Eppendorf twin.tec or Armadillo) – used for reagent processing	—	—	—
Shelf 3	Empty processing plate (Eppendorf twin.tec or Armadillo) – used for reagent processing	—	—	—
Shelf 2	New tip box	Hyb Buffer plate prepared on page 36	—	—
Shelf 1 (Bottom)	Empty tip box	Library Wash Buffer plate prepared on page 36	—	Empty tip box

2 Load the Bravo deck according to Table 24.

Table 24 Initial Bravo deck configuration for LibraryPrep_Avida_ILM runset

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	Empty red insert
5	Empty processing plate (Eppendorf twin.tec or Armadillo) – used for reagent processing
7	DNA sample plate prepared on page 35
9	Library prep master mix source plate prepared on page 39

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

Table 25 Initial BenchCel configuration for LibraryPrep_Avida_ILM runset

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	2 Tip boxes	—	—	—
3	2 Tip boxes	—	—	—
4	3 Tip boxes	—	—	—
6	4 Tip boxes	—	—	—
12	7 Tip boxes	—	—	—

Step 9. Run VWorks runset LibraryPrep_Avida_ILM

1 On the Avida VWorks form, under **Select protocol to execute**, select the **LibraryPrep_Avida_ILM** runset.

2 Under **Select labware for thermal cycling**, select the specific type of PCR plate to be used for the hybridization reactions.

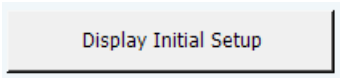
NOTE

If you select one of the semi-skirted plates for thermal cycling, VWorks will prompt you at the end of the runset when it is time to add the empty semi-skirted plate to Bravo deck position 4.

If you select one of the full-skirted plates (Eppendorf twin.tec or Armadillo) make sure that the sample plate containing the input DNA samples was prepared using that same plate type (see "Prepare the DNA sample plate (processing plate)" on page 35).

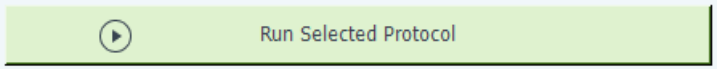
3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

4 Click **Display Initial Setup**.

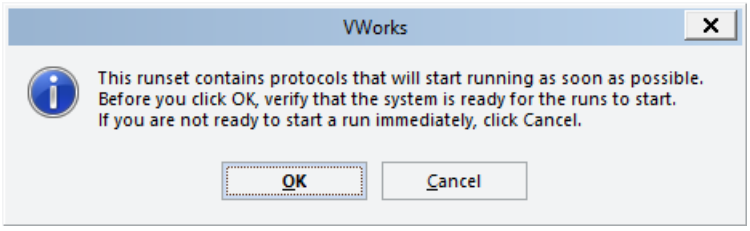


5 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



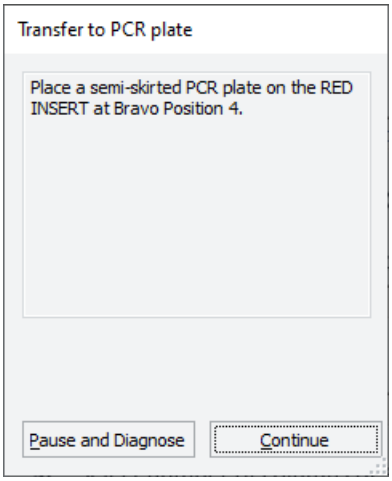
7 When ready to begin the run, click **OK** in the following window.



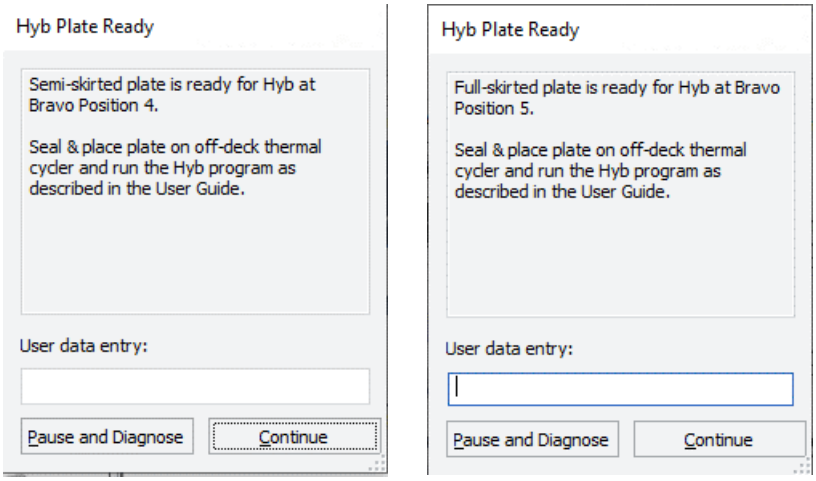
Running the LibraryPrep_Avida_ILM runset takes approximately 3 hours. During the runset, the NGS Workstation completes the PCR-free library preparation, including end-repair, dA-tailing, and ligation of the adaptor.

8 While the runset is running, pre-program the thermal cycler for the hybridization thermal cycler program as directed in Table 26 in "Step 10. Perform hybridization" on page 45.

9 If you selected a semi-skirted PCR plate in step 2, then VWorks prompts you near the end of the runset to add an empty PCR plate to position 4 of the Bravo deck. The prompt is shown below. After adding the plate, click **Continue**. The workstation then prepares the plate of hybridization reactions for transfer to the thermal cycler.



10 When prompted by VWorks that the Hyb plate is ready (the two possible prompts are shown below) at the completion of the LibraryPrep_Avida_ILM runset, remove the PCR plate from the Bravo deck as instructed in the prompt. This plate contains the hybridization reactions. After removing the plate, click **Continue**.



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

12 Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles. Proceed to "Step 10. Perform hybridization."

Step 10. Perform hybridization

1 While the LibraryPrep_Avida_ILM runset is in progress, pre-program the thermal cycler with the program in Table 26. Set the heated lid to 103°C. Start the program, then immediately pause the program, allowing the heated lid to reach temperature.

Table 26 Thermal cycler program for hybridization

Step	Temperature	Time
Step 1	98°C	2 minutes
Step 2	≥2.5°C/second ramp down to 60°C	
Step 3	60°C	60 minutes
Step 4	60°C	Hold

NOTE

The thermal cycler program in Table 26 uses a 60-minute hybridization which is sufficient for most applications. For panels >500 kb, such as the Avida DNA Discovery Cancer Panel, a longer hybridization time (up to 16 hours) may improve capture efficiency.

2 Transfer the sealed plate containing the hybridization reactions to the pre-programmed thermal cycler. Resume the pre-programmed thermal cycler program for hybridization (Table 26).

3 When the hybridization thermal cycler program has approximately 30 minutes remaining, start preparing for the Capture & Wash runset by proceeding to Chapter 5, "Capture and Washing" starting on page 46.

5 Capture and Washing

This chapter describes the steps for automated capture of targets captured during hybridization with the Avida DNA panel and washing of those targets.

The TargetCaptureDNA runset directs the NGS Workstation to complete the steps for:

- Capturing the library DNA hybrids from the first hybridization using Capture Beads
- Washing the bead-bound DNA

This chapter also describes the steps needed for preparation of the Capture Beads (upstream of target capture), which can be performed manually or using the automated protocol PrepareCaptureBeads.

NOTE Initiate the instructions in this chapter when the hybridization thermal cycler program initiated at the end of the previous chapter has approximately 30 minutes remaining. After starting the Target Capture DNA runset, the VWorks software prompts you when it is time to add the completed hybridization reaction plate to the Bravo deck.

This chapter uses the reagents listed in Table 27.

Table 27 Reagents for target capture and washing

Reagent	Usage Notes	Kit/Storage Location
Hyb Buffer (bottle)	Following use in previous chapter, keep at room temperature. Vortex before use. If precipitate is visible, reheat to 37°C before use.	Avida DNA Reagent Box 1, stored at –20°C
Capture Beads (tube with amber cap)	Thoroughly vortex to mix before use.	Avida Beads Box, stored at 4°C
Nuclease-Free Water (bottle)	—	Avida DNA and Duo Reagent Box 2, stored at Room Temperature
Hyb Wash Buffer 1 (bottle)	Heat the stock bottle at 50°C in water bath or heat block until use.	
Hyb Wash Buffer 2 (bottle)	—	
Resuspension Buffer (bottle)	—	

Capture Beads Preparation

The Capture Beads need to be washed before they can be used for target capture and washing. The Avida VWorks form supports two approaches for preparing washed Capture Beads: automated preparation and manual preparation.

Follow the instructions in Option 1: Prepare the Capture Beads source plate using automation to use automation to prepare the beads.

Follow the instructions in Option 2: Prepare the Capture Beads source plate manually to prepare the beads manually.

Option 1: Prepare the Capture Beads source plate using automation

This option uses the PrepareCaptureBeads automated protocol to wash the Capture Beads and prepare the Capture Beads source plate needed for the Target Capture DNA runset.

Step 1. Prepare the NGS Workstation for the PrepareCaptureBeads protocol

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 decontamination wipe.

Step 2. Prepare the PrepareCaptureBeads reagent source plate

1 Thoroughly vortex the Capture Beads, Hyb Wash Buffer 1 and Hyb Buffer as described in Table 27 on page 46.

CAUTION Hyb Wash Buffer 1 may become cloudy or form crystals during storage. Make sure that the solution clears during the 50°C heating. Thoroughly vortex or invert the stock bottle before use.

2 Using a processing plate (Eppendorf twin.tec or Armadillo), prepare the reagent source plate for the PrepareCaptureBeads protocol by adding the required reagents to the appropriate wells. Keep the plate at room temperature during and after preparation.

Table 28 lists the required volumes and well positions for each reagent based on the number of columns in the run. Figure 5 shows the configuration of the PrepareCaptureBeads source plate.

Table 28 Volumes (per well) for the PrepareCaptureBeads source plate based on number of columns in the run

Reagent	Position on Source Plate	Volume of master mix added per well of source plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
Capture Beads	Column 1 (A1-H1)	16 µL	24 µL	28 µL	36 µL	56 µL	112 µL
Hyb Wash Buffer 1 preheated to 50°C	Column 2 (A2-H2)	160 µL	160 µL	160 µL	160 µL	160 µL	160 µL
Hyb Wash Buffer 1 preheated to 50°C	Column 3 (A3-H3)	160 µL	160 µL	160 µL	160 µL	160 µL	160 µL
Hyb Buffer	Column 4 (A4-H4)	30 µL	39 µL	39 µL	50 µL	70 µL	130 µL

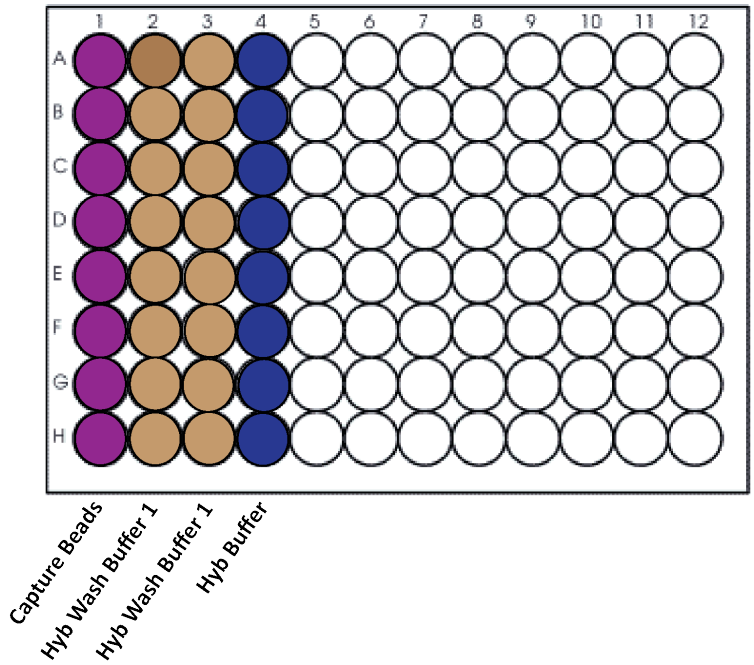


Figure 5 Configuration of the reagent source plate for protocol PrepareCaptureBeads

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

4 Centrifuge the plate at 50 × g for 1 second 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.

Step 3. Load the NGS Workstation for the PrepareCaptureBeads protocol

1 Load the Labware MiniHub according to Table 29. Use the plate orientations shown in Figure 4 on page 41.

Table 29 Initial MiniHub configuration for PrepareCaptureBeads protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	Empty processing plate (Eppendorf twin.tec or Armadillo)	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	—	—	—
Shelf 2	New tip box	—	—	—
Shelf 1 (Bottom)	Empty tip box	—	—	—

2 Load the Bravo deck according to Table 30.

Table 30 Initial Bravo deck configuration for PrepareCaptureBeads protocol

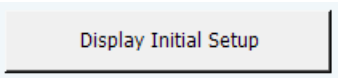
Location	Content
1	Empty waste plate (Agilent 2 mL square well)
5	PrepareCaptureBeads reagent source plate prepared on page 47

Step 4. Run the PrepareCaptureBeads protocol

1 On the Avida VWorks form, under **Select protocol to execute**, select the **PrepareCaptureBeads** protocol.

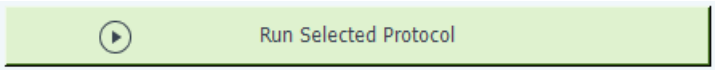
2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

3 Click **Display Initial Setup**.



4 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



6 When the PrepareCaptureBeads protocol is complete, the plate containing the washed Capture Beads is located at position 5. Remove the plate from the Bravo deck. *This plate serves as the Capture Beads source plate for the Target Capture DNA runset.*

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

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

Proceed immediately to "Target Capture/Washing" on page 52.

Option 2: Prepare the Capture Beads source plate manually

With this option, you manually wash the Capture Beads and prepare the Capture Beads source plate needed for the Target Capture DNA runset.

1 Thoroughly vortex the Capture Beads, Hyb Wash Buffer 1, and Hyb Buffer as described in Table 27 on page 46.

CAUTION Hyb Wash Buffer 1 may become cloudy or form crystals during storage. Make sure that the solution clears during the 50°C heating. Thoroughly vortex or invert the stock bottle before use.

2 Refer to Table 31 to determine the required volume of Capture Beads based on the number of columns in the run. Add that volume of Capture Beads to a 1.5-mL tube.

3 Place the tube of Capture Beads on the magnet for 1 minute or until the solution becomes clear. Remove and discard the supernatant. Remove the tube from the magnet.

4 Refer to Table 31 to determine the appropriate volume of preheated Hyb Wash Buffer 1 to use for washing the Capture Beads. Add that volume of Hyb Wash Buffer 1 to the tube of Capture Beads. Mix well by vortexing or by pipetting up and down at least 15–20 times.

Make sure the beads are fully resuspended and well mixed. When working with wash volumes >100 µL, mixing by pipetting is more effective than vortexing.

5 Briefly spin the tube of Capture Beads, then place the tube on the magnet for 1 minute or until the solution becomes clear. Remove and discard the supernatant.

6 Perform an additional wash by repeating step 4 through step 5 for a total of two washes.

Place the Hyb Wash Buffer 1 back on the 50°C heat block or water bath.

7 Remove the tube of Capture Beads from the magnet. Resuspend the beads in the appropriate volume of Hyb Buffer listed in Table 31. Mix by pipetting up and down 15–20 times (avoid creating excess bubbles).

Table 31 Volumes for preparing washed Capture Beads in a 1.5-mL tube or conical tube

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
Capture Beads	8 µL	128 µL	192 µL	224 µL	288 µL	448 µL	896 µL
Hyb Wash Buffer 1 preheated to 50°C	100 µL	100 µL	100 µL	200 µL	200 µL	300 µL	600 µL
Hyb Buffer	8 µL	128 µL	192 µL	224 µL	288 µL	448 µL	896 µL

8 In the wells of a processing plate (Eppendorf twin.tec or Armadillo), add 8 µL of washed Capture Beads to each well of each column included in the run. *This plate serves as the Capture Beads source plate for the Target Capture DNA runset.*

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

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

Proceed immediately to "Target Capture/Washing" on page 52.

Target Capture/Washing

Step 1. Prepare the NGS Workstation for the Target Capture DNA runset

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 decontamination wipe.
3. Place the silver Deep Well plate insert on position 6 of the Bravo deck. This insert is required to facilitate heat transfer to the Deep Well source plate wells during the Target Capture DNA runset. When loading a source plate on the silver insert, make sure the plate is seated properly to ensure proper heat transfer.
4. Pre-set the temperature of Bravo deck position 4 to 63°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
5. Pre-set the temperature of Bravo deck position 6 to 50°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.

Step 2. Prepare plates for target capture and washing

The following plates need to be prepared prior to starting the Target Capture DNA runset. Store plates at room temperature after preparation.

Prepare the Hyb Wash Buffer 1 plate (Agilent Deep Well plate)

1. In the wells of an Agilent Deep Well plate, add 300 µL of preheated Hyb Wash Buffer 1 to each well of each column included in the run.
2. Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
3. Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

Prepare the Hyb Wash Buffer 2 plate (Agilent Deep Well plate)

1. In the wells of an Agilent Deep Well plate, add 160 µL of Hyb Wash Buffer 2 to each well of each column included in the run.
2. Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.
3. Centrifuge the plate for 30 seconds to drive the well contents off the walls and plate seal and to eliminate any bubbles.

Prepare the Resuspension Buffer plate (processing plate)

1 Pour the Resuspension Buffer from the stock bottle into a reagent reservoir.

2 Use a multichannel pipette to transfer 24 µL of Resuspension Buffer from the reservoir to the wells of a processing plate (Eppendorf twin.tec or Armadillo), starting with column 1. Add the Resuspension Buffer to each column included in the run.

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

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

5 Transfer any remaining Resuspension Buffer from the reagent reservoir back into the stock bottle.

Step 3. Load the NGS Workstation for the Target Capture DNA runset

1 Load the Labware MiniHub according to Table 32. Use the plate orientations shown in Figure 4 on page 41.

Table 32 Initial MiniHub configuration for TargetCaptureDNA runset

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	—	—	—	—
Shelf 4	Resuspension Buffer plate prepared on page 53	—	—	—
Shelf 3	Empty processing plate (Eppendorf twin.tec or Armadillo)	—	—	—
Shelf 2	—	—	Hyb Wash Buffer 2 plate prepared on page 52	—
Shelf 1 (Bottom)	—	—	—	Empty tip box

2 Load the Bravo deck according to Table 33.

Table 33 Initial Bravo deck configuration for TargetCaptureDNA runset

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
4	Empty red insert
5	Capture Bead source plate (prepared in "Capture Beads Preparation")
6	Hyb Wash Buffer 1 plate prepared on page 52, seated in silver insert
7	Empty at start of runset (add hybridization plate from the thermal cycler when prompted by VWorks)

3 Load the BenchCel Microplate Handling Workstation according to Table 34.

Table 34 Initial BenchCel configuration for TargetCaptureDNA runset

No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	2 Tip boxes	—	—	—
3	2 Tip boxes	—	—	—
4	3 Tip boxes	—	—	—
6	4 Tip boxes	—	—	—
12	7 Tip boxes	—	—	—

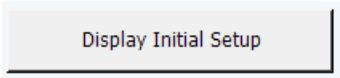
Step 4. Run the Target Capture DNA runset

NOTE Approximately 20 minutes after starting the runset, VWorks opens a prompt to transfer the hybridization plate from the thermal cycler to the Bravo deck. Shortly after, VWorks opens another prompt to remove the hybridization plate from the Bravo deck. Make sure an operator is present to perform these actions. The remainder of the runset takes approximately 30 minutes to complete.

1 On the Avida VWorks form, under **Select protocol to execute**, select the **TargetCaptureDNA** runset.

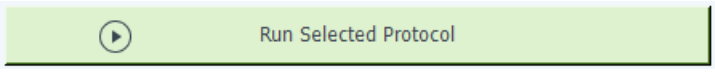
2 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

3 Click **Display Initial Setup**.

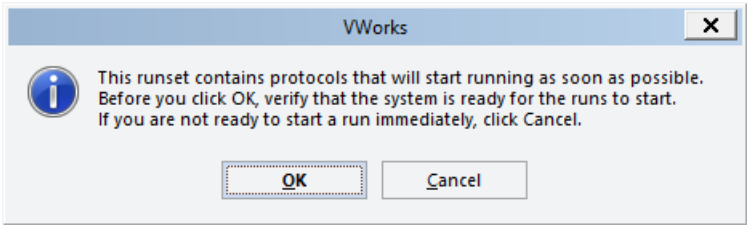


4 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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

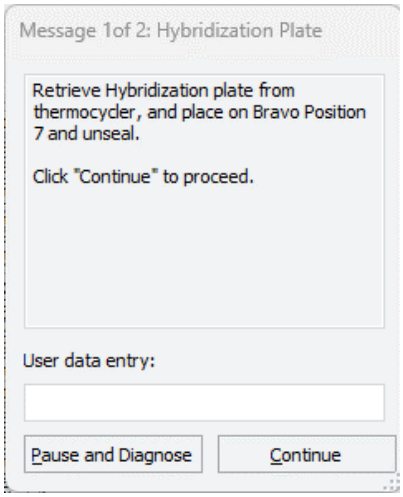


6 If the following prompt opens, click **OK** to continue.

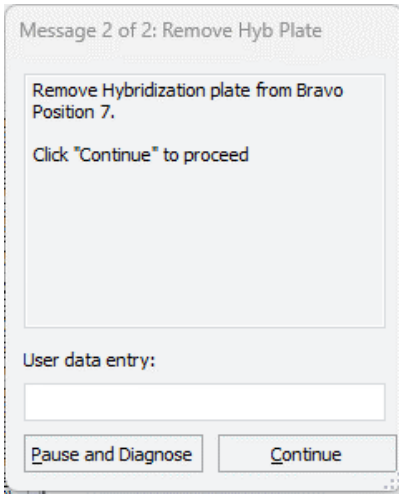


7 When prompted by VWorks as shown below, transfer the hybridization plate from the thermal cycler to position 7 of the Bravo deck. After adding the plate, click **Continue**.

NOTE If the hybridization thermal cycler program has not yet reached the 60°C hold step, wait until that step is reached before transferring the plate from the thermal cycler to the Bravo deck.



8 When prompted by VWorks for a second time, as shown below, remove and discard the now empty hybridization plate from position 7 of the Bravo deck. After removing the plate, click **Continue**.



9 When the Target Capture DNA runset is complete, the plate containing the resuspended Capture Beads with captured DNA is located in the red insert at position 4 of the Bravo deck. Transfer the plate to ice.

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

To avoid bead settling, proceed immediately to Chapter 6, "Indexing PCR" starting on page 57. The plate of resuspended Capture Beads with captured DNA will be loaded on the Bravo deck (see Table 38 on page 60) for the PCR_Avida_ILM protocol.

6 Indexing PCR

This chapter describes the steps for automated plate setup for the indexing PCR reactions.

The PCR automation protocol (PCR_Avida_ILM) directs the NGS Workstation to complete the steps for preparing the PCR plate. After the PCR plate is prepared by the workstation, VWorks prompts you to transfer the plate to a thermal cycler for amplification. The PCR reactions use index primer pairs to generate amplified, indexed libraries for targeted DNA sequencing.

This chapter uses the reagents listed in Table 35.

Table 35 Reagents for indexing PCR

Reagent	Usage Notes	Kit/Storage Location
2X Amplification Mastermix (bottle)	Thaw on ice.	Avida DNA Reagent Box 1, stored at –20°C
Avida Index Primer Pairs	Primer pairs are provided in a 96-well plate. Thaw plate on ice.	Stored at –20°C
Nuclease-Free Water (bottle)	—	Avida DNA and Duo Reagent Box 2, stored at Room Temperature

CAUTION Take precautions to avoid amplicon contamination during setup of the indexing PCR reactions. Review "Important practices for preventing contamination" on page 11.

Step 1. Prepare the NGS Workstation for the PCR_Avida_ILM protocol

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

2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.

3 Pre-set the temperature of Bravo deck position 4 to 20°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.

4 Pre-set the temperature of Bravo deck position 6 to 4°C using the Inheco Multi TEC control touchscreen. Bravo deck position 6 corresponds to CPAC 2, position 2 on the Multi TEC control touchscreen.

5 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.

Step 2. Prepare the 2X Amplification Mastermix source plate

The 2X Amplification Mastermix source plate is a **processing plate** containing the 2X Amplification Mastermix used in the PCR_Avida_ILM protocol.

1 Using a processing plate (Eppendorf twin.tec or Armadillo), prepare the 2X Amplification Mastermix source plate for the PCR_Avida_ILM protocol by adding the required volume of 2X Amplification Mastermix to the appropriate wells, as indicated in Table 36. Keep the plate on ice during and after preparation.

   For runs with 1, 2, 3, 4, 6 or 12 columns, all wells in column 1 need to be filled with the volume of 2X Amplification Mastermix indicated in Table 36. For runs with 12 columns, all wells of column 2 also need to be filled with the indicated volume. The final configuration of the source plate is shown in Figure 6.

   At the end of the PCR_Avida_ILM protocol, you can retain the source plate for use in the TS_D1000 protocol. See "Prepare the workstation and Sample Buffer source plate" on page 68.

Table 36 Preparation of the 2X Amplification Mastermix source plate for PCR_Avida_ILM protocol

Master Mix Solution	Position on Source Plate	Volume of master mix added per well of source plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
2X Amplification Mastermix	Column 1 (A1-H1)	29.0 µL	54.0 µL	79.0 µL	104.0 µL	154.0 µL	154.0 µL
	Column 2 (A2-H2)	—	—	—	—	—	154.0 µL

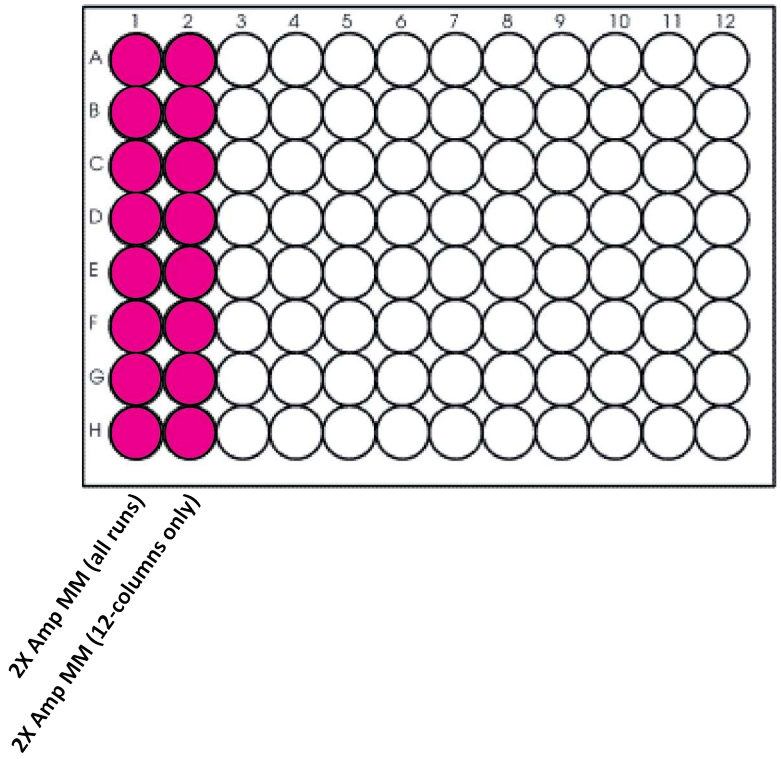


Figure 6 Configuration of the 2X Amplification Mastermix source plate (Eppendorf twin.tec or Armadillo plate) for protocol PCR_Avida_ILM. For all runs, the 2X Amplification Mastermix is distributed to all wells of column 1. For runs with 12 columns, the 2X Amplification Mastermix is also distributed to all wells of column 2.

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

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

Step 3. Prepare the Index Primer Pairs plate

The Index Primer Pairs plate is a **PCR plate** containing the Avida Index Primer Pairs used in the PCR_Avida_ILM protocol.

1 Using a multichannel pipette, transfer 5 µL of each Avida Index Primer Pair from the 96-well plate in which the primer pairs are provided into the PCR plate to be used for the indexing PCR thermal cycling. Make sure to maintain the same well location for each primer pair when transferring to the PCR plate. Keep the PCR plate on ice.

NOTE

Index primer pairs are designed to be used in groups of 8. That is, pairs 1–8 are compatible with each other, as are pairs 9–16, 17–24, etc. For samples to be sequenced in the same lane, the best practice is to use primer pairs within a compatible set (e.g., 1–8). If you use primer pairs from across different compatibility sets, then you need to confirm that the selected pairs meet the compatibility requirements for Illumina sequencing. Specifically, make sure that none of the 16 nucleotide positions (8 on forward primer and 8 on reverse primer) contain only A and C nucleotides or only T and G nucleotides across all indexes in the set.

Step 4. Load the NGS Workstation for the PCR_Avida_ILM protocol

1 Load the Labware MiniHub according to Table 37, using the plate orientations shown in Figure 4 on page 41.

Table 37 Initial MiniHub configuration for PCR_Avida_ILM protocol

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

2 Load the Bravo deck according to Table 38.

Table 38 Initial Bravo deck configuration for PCR_Avida_ILM protocol

Location	Content
4	Resuspended Capture Beads with captured DNA in processing plate (unsealed) seated in red insert
6	Index Primer Pairs plate prepared on page 60 (seated in red insert if plate is semi-skirted)
9	2X Amplification Mastermix source plate prepared on page 58

3 Load the BenchCel Microplate Handling Workstation according to Table 39.

Table 39 Initial BenchCel configuration for PCR_Avida_ILM protocol

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

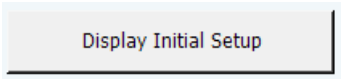
Step 5. Run VWorks protocol PCR_Avida_ILM

1 On the Avida VWorks form, under **Select protocol to execute**, select the **PCR_Avida_ILM** protocol.

2 Under **Select labware for thermal cycling**, select the specific type of PCR plate used at position 6 of the Bravo deck.

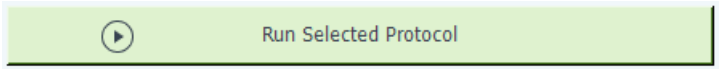
3 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

4 Click **Display Initial Setup**.



5 Verify that the NGS Workstation has been set up as displayed on the right side of the form.

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



Running the protocol takes approximately 15 minutes. Once complete, the PCR reactions – containing captured DNA, 2X Amplification Mastermix, and Avida Index Primer Pairs – are located in the PCR plate at position 6 of the Bravo deck.

7 Proceed to "Step 6. Perform PCR cycling" below to start preparing the thermal cycler for PCR cycling.

Step 6. Perform PCR cycling

1 While the PCR_Avida_ILM protocol is in progress, pre-program the thermal cycler to run the program shown in Table 40. Set the heated lid to 103°C. Start the program, then immediately pause the program, allowing the heated lid to reach temperature.

Table 40 Thermal cycler program for first indexing PCR

Step	Number of Cycles	Temperature	Time
Initial denaturation	1	98°C	45 seconds
Amplification stage 1	5	98°C	10 seconds
		63°C	30 seconds
		72°C	30 seconds
Amplification stage 2	Variable, see Table 41 for recommendations	98°C	10 seconds
		72°C	1 minute
Final extension	1	72°C	1 minute
Final hold	1	4°C	Hold

Table 41 Indexing PCR cycle number recommendations for Amplification stage 2

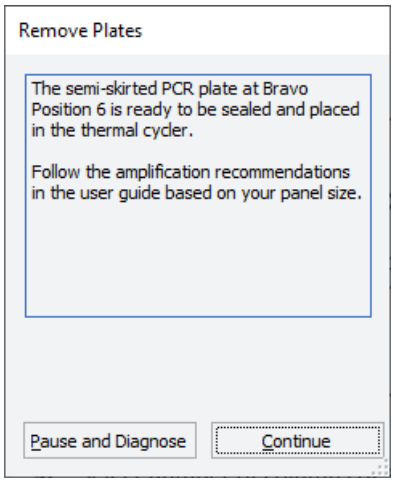
Probes size of panel	Number of cycles based on 10 ng cfDNA input -- See Note below for other sample types/quantities
>100 kb	16 or fewer • Avida DNA Discovery Cancer Panel: 11 cycles • Avida DNA Onco LB Plus Panel: 13 cycles • Avida DNA Expanded Cancer Panel: 14 cycles • Avida DNA Onco LB Panel: 14 cycles • Avida DNA Lymphoma Panel: 16 cycles
>50 kb to 100 kb	17
10 kb to 50 kb	18 • Includes the Avida DNA Focused Panel
<10 kb	19

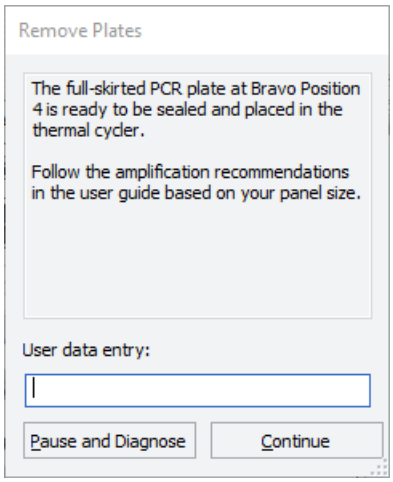
NOTE

The number of cycles recommended in Table 41 are based on a sample input of 10 ng of cfDNA. If using other sample types or input quantities, you may need to optimize the number of cycles using the points below for guidance.

- High-quality gDNA samples typically require 1–2 cycles more than cfDNA samples of the same input quantity.
- Low-quality gDNA samples (e.g., FFPE-derived gDNA) may require more cycles than high-quality gDNA samples. The number of additional cycles depends on the degree of sample degradation, with greater degradation necessitating more cycles.
- A sample input >50 ng typically requires 1–2 fewer cycles than a sample input of 10 ng.

2 When prompted by VWorks that the PCR plate is ready (the two possible prompts are shown below), remove the PCR plate from the Bravo deck as instructed in the prompt. Seal the plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 seconds.





3 Centrifuge the sealed plate at 50 × g for 1 second to drive the well contents off the walls and plate seal and to eliminate any bubbles

4 Place the plate in the thermal cycler. Resume the thermal cycler program in Table 40 on page 62.

5 While the indexing PCR thermal cycler program is running, start preparing for Library Purification by proceeding to Chapter 7, “Library Purification, Quality Assessment, and Sample Pooling” starting on page 64.

Stopping Point If you do not continue to the next step, store the indexed libraries at 4°C overnight by leaving the plate in the thermal cycler at the 4°C hold step.

7 Library Purification, Quality Assessment, and Sample Pooling

This chapter describes the steps for automated library purification, quality analysis plate setup, and optional sample pooling.

The Library Cleanup automation protocol (AMPureXP_Cleanup) is used to purify the libraries. The TapeStation DNA 1000 automation protocol (TS_D1000) directs the workstation to set up the analysis plate for the DNA 1000 assay on the TapeStation. The automation protocol for sample pooling (Aliquot_Captures) can be used to pool samples prior to sequencing.

This chapter uses the reagents listed in Table 42.

Table 42 Reagents for library purification and quality assessment

Reagent	Usage Notes	Kit/Storage Location
AMPure XP Beads	Equilibrate at room temperature for 30 minutes.	Not provided
100% ethanol	—	Not provided
1X Low TE Buffer	—	Not provided
Qubit dsDNA HS or BR Assay Kit (optional)	—	Not provided
Nucleic acid analysis kit	—	Not provided

Library Purification

For library purification, the NGS Workstation runs the AMPureXP_Cleanup protocol to transfer AMPure XP beads to the indexed DNA sample plate and then collect and wash the bead-bound DNA.

Step 1. Prepare the AMPure XP bead plate (processing plate)

1. Verify that the AMPure XP bead suspension is at room temperature.
2. Mix the bead suspension well so that the reagent appears homogeneous and consistent in color. Do not freeze.
3. In the wells of a processing plate (Eppendorf twin.tec or Armadillo), add 45 µL of bead suspension each well of each column included in the run.

   You can use a reagent reservoir and multichannel pipette to perform this step.

Step 2. Prepare the 1X Low TE Buffer plate (processing plate)

1. In the wells of a processing plate (Eppendorf twin.tec or Armadillo), add 30 µL of 1X Low TE Buffer to each well of each column included in the run.
2. Seal the sample plate using the PlateLoc Thermal Microplate Sealer, with sealing settings of 165°C and 1.0 sec.

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

Step 3. Prepare the NGS Workstation

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 decontamination wipe.
3. Pre-set the temperature of Bravo deck position 4 to 37°C using the Inheco Multi TEC control touchscreen, as described in Setting the Temperature of Bravo Deck Heat Blocks. Bravo deck position 4 corresponds to CPAC 2, position 1 on the Multi TEC control touchscreen.
4. Turn on the ThermoCube, set to 0°C, at position 9 of the Bravo deck. Be sure that the chiller reservoir contains at least 300 mL of 25% ethanol.
5. Prepare an Agilent deep well reservoir containing 50 mL of freshly-prepared 80% ethanol.
6. Load the Labware MiniHub according to Table 43, using the plate orientations shown in Figure 4 on page 41.

Table 43 Initial MiniHub configuration for AMPureXP_Cleanup protocol

Vertical Shelf Position	Cassette 1	Cassette 2	Cassette 3	Cassette 4
Shelf 5 (Top)	AMPure XP bead plate prepared on page 65	—	—	—
Shelf 4	—	—	—	—
Shelf 3	—	Empty processing plate	—	—
Shelf 2	—	1X Low TE Buffer plate prepared on page 65		—
Shelf 1 (Bottom)	—	80% ethanol reservoir prepared on page 65	—	Empty tip box

7 Load the Bravo deck according to Table 44.

Table 44 Initial Bravo deck configuration for AMPureXP_Cleanup protocol

Location	Content
1	Empty waste plate (Agilent 2 mL square well)
9	Amplified DNA libraries in unsealed PCR plate; seated in a red insert if using a semi-skirted plate (PCR plate type must be specified on setup form under step 2)

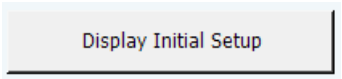
8 Load the BenchCel Microplate Handling Workstation according to Table 45.

Table 45 Initial BenchCel configuration for AMPureXP_Cleanup protocol

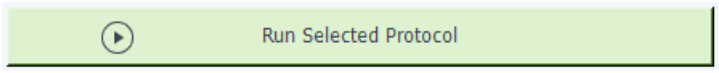
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	2 Tip boxes	—	—	—
4	2 Tip boxes	—	—	—
6	3 Tip boxes	—	—	—
12	5 Tip boxes	—	—	—

Step 4. Run VWorks protocol AMPureXP_Cleanup

1. On the Avida DNA VWorks form, under **Select protocol to execute**, select the **AMPureXP_XT_HS2_ILM (Post-Capture PCR)** protocol.
2. Under **Select labware for thermal cycling**, select the specific type of PCR plate containing the amplified libraries at position 9.
3. Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.
4. Click **Display Initial Setup**.



5. Verify that the NGS workstation has been set up as displayed on the right side of the form.
6. When verification is complete, click **Run Selected Protocol**.



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

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

Library Quantity and Quality Assessment

1 (Optional) Measure the libraries using a fluorescence-based method for DNA quantification, such as the Qubit dsDNA HS Assay Kit or Qubit dsDNA BR Assay Kit. Follow the manufacturer's instructions for the instrument and assay kit.

2 Perform post-capture library analysis using one of two options.

- Option 1: Prepare the analytical assay plate using automation (protocol TS_D1000) and perform analysis on Agilent 4200 TapeStation. See "Option 1: Analysis using an Agilent 4200 TapeStation and D1000 ScreenTape" on page 68.
- Option 2: Prepare the analytical samples manually and perform analysis on Agilent 4200 or 4150 TapeStation or Agilent 5200 Fragment Analyzer. See "Option 2: Analysis using an equivalent platform (non-automated)" on page 73.

Option 1: Analysis using an Agilent 4200 TapeStation and D1000 ScreenTape

This section describes use of automation protocol TS_D1000 to prepare the D1000 assay sample plate by combining 2 µL of each DNA sample with 6 µL of D1000 sample buffer. Afterward, you transfer the prepared assay plate to the 4200 TapeStation instrument for analysis. For more information to do this step, see the Agilent D1000 Assay Quick Guide for 4200 TapeStation System.

Allow the reagents used with either analysis system to equilibrate to room temperature for 30 minutes prior to use.

Prepare the workstation and Sample Buffer source plate

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

2 Gently wipe down the Labware MiniHub, Bravo deck, and BenchCel with a decontamination wipe.

3 Turn off the ThermoCube device (see page 22) to restore position 9 of the Bravo deck to room temperature.

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

5 For the Sample Buffer source plate, use the same **processing plate** (Eppendorf twin.tec or Armadillo) that was used as the 2X Amplification Mastermix source plate or 2X Methyl Amplification Mastermix source plate in the PCR indexing protocol. Prepare the Sample Buffer source plate at room temperature. Add the volume of D1000 Sample Buffer indicated in Table 46 to each well of column 3 of the plate. The final configuration of the Sample Buffer source plate is shown in Figure 7.

Table 46 Preparation of the Sample Buffer Source Plate for TS_D1000 protocol

Solution	Position on Source Plate	Volume of Sample Buffer added per Well of Processing Plate					
		1-Column Runs	2-Column Runs	3-Column Runs	4-Column Runs	6-Column Runs	12-Column Runs
D1000 Sample Buffer	Column 3 (A3-H3)	11.0 µL	17.0 µL	23.0 µL	29.0 µL	42.0 µL	80.0 µL

CAUTION Make sure to add the D1000 Sample Buffer to column 3 of the source plate.

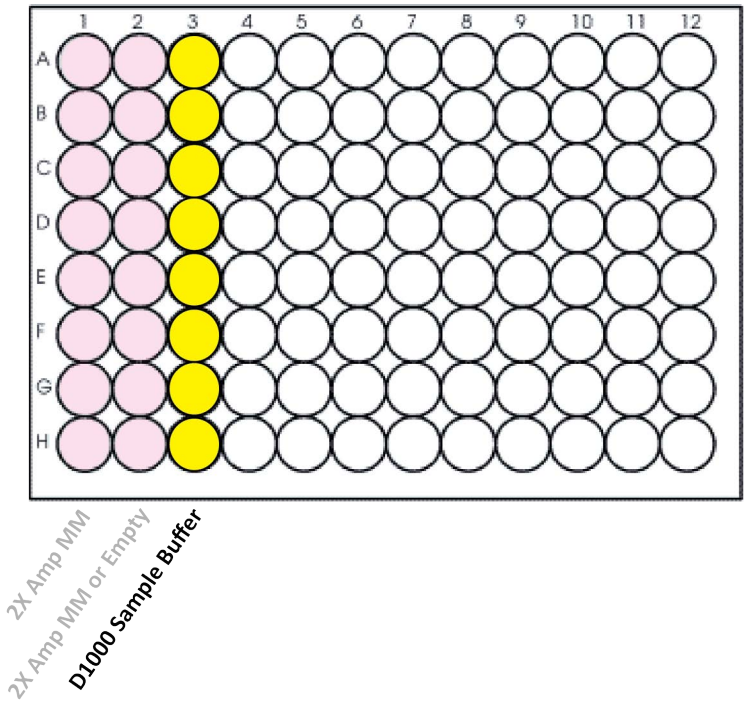


Figure 7 Configuration of the Sample Buffer source plate for protocol TS_D1000. Columns 1–2 were used in the previous protocol.

Load the Agilent NGS Workstation

6 Load the Labware MiniHub according to Table 47, using the plate orientations shown in Figure 4 on page 41.

Table 47 Initial MiniHub configuration for TS_D1000 protocol

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

7 Load the Bravo deck according to Table 48.

Table 48 Initial Bravo deck configuration for TS_D1000 protocol

Location	Content
4	Amplified post-capture libraries in processing plate (unsealed)
6	Empty Eppendorf twin.tec plate
9	Sample Buffer source plate containing D1000 Sample Buffer in Column 3

CAUTION

To prevent damage to the Agilent 4200 TapeStation instrument and the Agilent NGS Workstation, use only the specified Eppendorf twin.tec plates (Eppendorf p/n 951020401 or 951020619) for automated assay plate preparation.

The Agilent 2200 TapeStation system does not support use of the Eppendorf twin.tec plates. Do not load sample plates prepared using the automated protocol in the 2200 TapeStation instrument.

8 Load the BenchCel Microplate Handling Workstation according to Table 49.

Table 49 Initial BenchCel configuration for TS_D1000 protocol

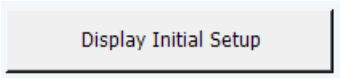
No. of Columns Processed	Rack 1	Rack 2	Rack 3	Rack 4
1	1 Tip box	—	—	—
2	1 Tip box	—	—	—
3	1 Tip box	—	—	—
4	1 Tip box	—	—	—
6	1 Tip box	—	—	—
12	1 Tip box	—	—	—

Run VWorks protocol TS_D1000

9 On the Avida VWorks form, under **Select protocol to execute**, select the **TS_D1000** protocol.

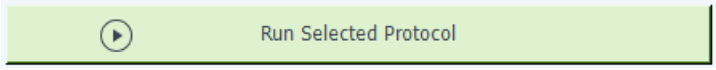
10 Select the number of columns of samples to be processed. Runs must include 1, 2, 3, 4, 6, or 12 columns.

11 Click **Display Initial Setup**.



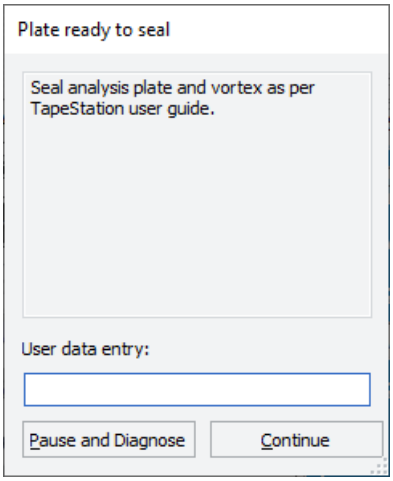
12 Verify that the NGS workstation has been set up as displayed on the right side of the form.

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



Running the TS_D1000 protocol takes approximately 15 minutes. Once removal of analytical samples is complete, remove the primary DNA library sample plate from position 4, seal the plate, and keep on ice until the samples are pooled for sequencing on page 74.

14 When prompted by VWorks as shown below, remove the Eppendorf twin.tec plate containing the analytical samples from position 6 of the Bravo deck, then click **Continue**. Seal the assay plate with a foil seal and vortex the sealed plate as directed in the Agilent D1000 Assay Quick Guide for 4200 TapeStation System.



CAUTION

To prevent damage to the Agilent 4200 TapeStation instrument, use only the specified 96-well plate foil seals (Agilent p/n 5067-5154).

For accurate quantitation, make sure that you thoroughly mix the combined DNA and sample buffer on a vortex mixer for 1 minute, then spin briefly to collect the liquid.

Run the D1000 Assay and analyze the data

15 Load the analytical sample plate, the D1000 ScreenTape, and loading tips into the TapeStation as instructed in the instrument user manual. Start the run.

16 Determine the concentration of each library by integrating under the entire peak.

See Table 50 for fragment size distribution guidelines. Representative electropherograms generated using the TapeStation system are provided in Figure 8 (cfDNA sample) and Figure 9 (gDNA sample) to illustrate typical results.

Table 50 Library qualification guidelines

Input DNA type	Expected DNA fragment size peak position
cfDNA	320 bp (see Figure 8 for sample electropherogram)
Fragmented gDNA*	300–450 bp (see Figure 9 for sample electropherogram)

* Refers to high-quality gDNA samples. For gDNA derived from FFPE samples, the fragment sizes may be smaller.

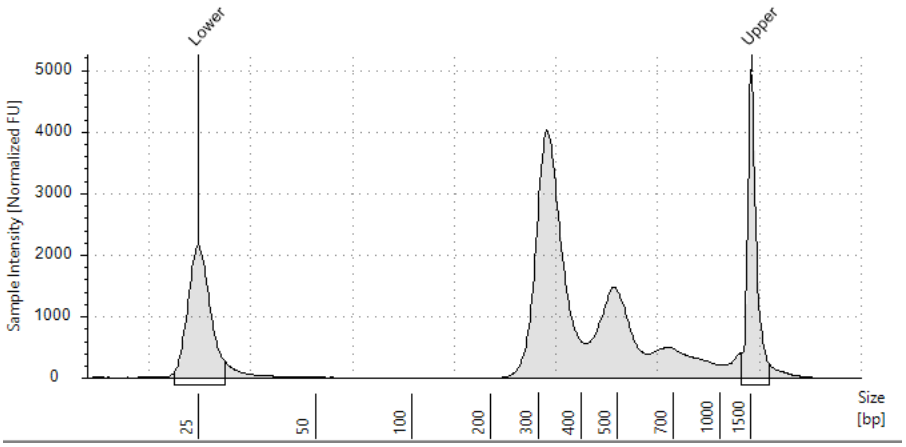


Figure 8 Library prepared from 10 ng of a cfDNA sample hybridized with the Avida DNA Focused Cancer Panel and analyzed using a D1000 ScreenTape assay.

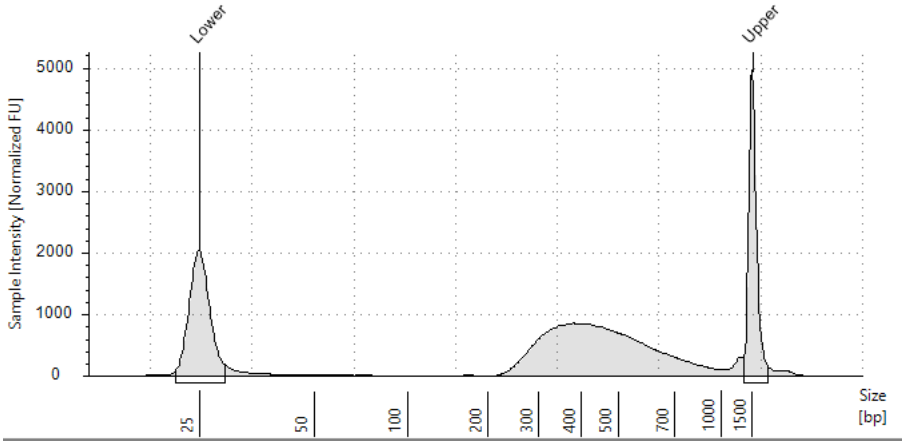


Figure 9 Library prepared from 10 ng of a high-quality, fragmented gDNA sample hybridized with the Avida DNA Focused Cancer Panel and analyzed using a D1000 ScreenTape assay.

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

Option 2: Analysis using an equivalent platform (non-automated)

Using manual preparation of the analytical samples, you can analyze the DNA samples on an Agilent 5200 Fragment Analyzer or Agilent 4200/4150 TapeStation. Electropherograms obtained using one of these other analysis platforms are expected to show fragment size profiles similar to those shown for the Agilent 4200 TapeStation (see Figure 8 through Figure 9). Verify that the electropherogram shows the expected DNA fragment size peak position (see Table 50 for guidelines). Table 51 includes links to assay instructions.

Table 51 Post-capture library analysis options

Analysis platform	Assay used at this step (links go to assay instructions)	Amount of library sample to analyze
Agilent 4200/4150 TapeStation system	D1000 ScreenTape	1 µL of sample mixed with 3 µL of D1000 sample buffer*
Agilent 5200, 5300, or 5400 Fragment Analyzer system	NGS Fragment Kit (1-6000 bp) Small Fragment Kit (1-1500 bp) HS NGS Fragment Kit (1-6000 bp) HS Small Fragment Kit (1-1500 bp)	2 µL of sample

* If the sample input for the workflow was near the top of the input range of 100 ng, you may need to further dilute the sample prior to analysis to make sure the quantity is in the dynamic range for the TapeStation system.

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

Sample Pooling for Multiplexed Sequencing (optional)

NOTE 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.

Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool using one of the two options described below. Option 2 can use the Aliquot_Captures automation protocol to pool samples.

Option 1: Pool samples at equal volumes (non-automated)

Dilute each indexed library to be pooled to the same final concentration (typically 4 nM to 15 nM, or the concentration of the most dilute sample) using 1X Low TE Buffer. This dilution step is performed by manually pipetting the 1X Low TE Buffer directly into the wells of the source plate. Then, combine equal volumes of all libraries to create the final pool in the destination plate.

Option 2: Pool samples at varying volumes (automated or non-automated)

Starting with indexed libraries at different concentrations, add the appropriate volume of each library to the destination well (either manually or using the Aliquot_Captures automation protocol) to achieve equimolar concentration in the pool. Then, adjust the pool to the desired final volume by adding the appropriate volume of 1X Low TE Buffer to each well. This volume adjustment is performed by manually pipetting the 1X Low TE Buffer directly into the wells of the destination plate. The formula below is provided for determination of the amount of each indexed sample to add to the pool.

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

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 (typically 4 nM–15 nM or the concentration of the most dilute sample)

is the number of indexes, and

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

Table 52 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 DNA.

Table 52 Example of volume calculation for total volume of 20 µL at 10 nM concentration

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

Pool samples for multiplexed sequencing using automation (optional for Option 2)

The instructions below are for Option 2: Pool samples at varying volumes (automated or non-automated). Alternatively, you can perform this method using an entirely manual approach rather than with the Aliquot_Captures automation protocol.

1. Create a CSV (comma separated value) file with the headers shown in Figure 10. The header text must not contain spaces. The table may be created using a spreadsheet application, such as Microsoft Excel software, and then saved in CSV format. The file must include rows for all 96 wells of the plate.
2. Enter the information requested in the header for each DNA sample. Figure 10 shows an example spreadsheet.
   - In the SourceBC field, enter the sample plate description or barcode. The SourceBC field contents must be identical for all rows.
   - In the SourceWell field, enter each well position on the source plate containing an amplified and captured indexed library that needs to be added to a pool. Use the Eppendorf twin.tec plate containing the purified indexed libraries as the source plate.
   - In the DestinationWell field, enter the well position on the destination plate for the pool.
   - In the Volume field, enter the volume (in µL) of each indexed library to be transferred from the source well to the destination well. The volume for each library is calculated from its concentration.
   - For all empty wells on the source plate, delete the corresponding rows in the CSV file.

	A	B	C	D
1	SourceBC	SourceWell	DestinationWell	Volume
2	abc	A1	A1	4.711292
3	abc	B1	A1	6.37105
4	abc	C1	A1	7.000448
5	abc	D1	A1	3.81144
6	abc	E1	A1	9.539072
7	abc	F1	A1	7.802747
8	abc	G1	A1	8.835171
9	abc	H1	A1	6.313131
10	abc	A2	A1	5.976286
11	abc	B2	A1	6.601183
12	abc	C2	A1	7.14449
13	abc	D2	A1	5.66431

Figure 10 Sample spreadsheet for option 2

NOTE

You can find a sample spreadsheet in the directory **C:\VWorks Workspace\NGS Option B\Avida_DNA_v.B1.3.x.x\Aliquot Input File Templates\Aliquot_Captures_Template.csv** (where x.x is the version number).

The Aliquot_Captures_template.csv file may be copied and used as a template for creating the CSV files for each Aliquot_Captures protocol run. If you are using the sample file as a template for runs with fewer than 12 columns, be sure to delete rows for any unused columns on the plate.

3 Load the CSV file onto the PC containing the VWorks software into a suitable directory, such as **C:\VWorks Workspace\NGS Option B\Avida_DNA_v.B1.3.x.x\Aliquot Input File Templates**.

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

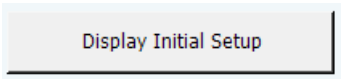
5 Load the Bravo deck according to Table 53.

Table 53 Initial Bravo deck configuration for Aliquot_Captures protocol

Location	Content
5	Empty Agilent Deep Well plate
6	Empty tip box
8	New tip box
9	Purified amplified indexed libraries in processing plate

6 On the Avida DNA VWorks form, under **Select protocol to execute**, select the **Aliquot_Captures** protocol.

7 Click **Display Initial Setup**.



8 Upload the CSV file created in step 1 through step 3 (page 75 to 76).

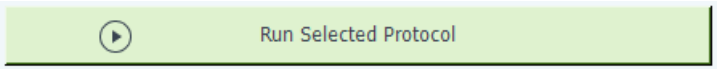
a Click the "**...**" browse button below **Select Aliquot Input File** to open a directory browser window.

b Browse to the location where you saved the CSV file. Select the file and click **Open**.

The directory browser window closes, returning you to the Avida DNA setup form. The selected file location is listed in the field below **Select Aliquot Input File**.

9 Verify that the NGS workstation has been set up as displayed on the right side of the form.

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



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

11 Remove the destination plate from the Bravo deck.

12 Add the appropriate volume of 1X Low TE Buffer to each well to bring the pool to the necessary DNA concentration for sequencing.

8
Sequencing and NGS Analysis

This chapter contains guidance on library sequencing and analysis. Refer to your specific Illumina sequencer's user guide for specific instructions on how to perform sequencing.

Step 1. Prepare the sequencing samples

The final Avida library pool is ready for sequencing using standard Illumina paired-end primers and chemistry. Each fragment in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform (Figure 11).

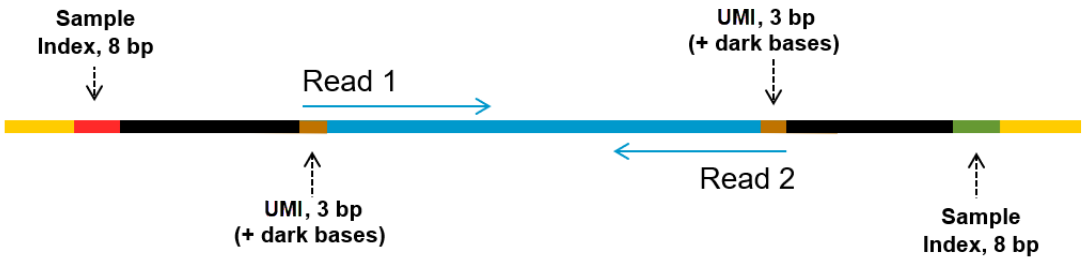


Figure 11 Content of Avida sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), unique dual sample indexes (red and green), duplex UMIs (brown), and the library PCR primers (yellow).

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit and sequence the libraries using an Illumina instrument. Consult Illumina's documentation for kit configuration and seeding concentration guidelines.

Seeding concentration and cluster density may need to be optimized based on the DNA fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range provided by Illumina. Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Step 2. Sequence the libraries

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. Table 54 provides guidelines for use of several Illumina instrument and chemistry combinations suitable for processing Avida target-enriched NGS libraries. For other Illumina NGS platforms, consult Illumina's documentation for kit configuration and seeding concentration guidelines.

Seeding concentration and cluster density may also need to be optimized based on the fragment size range for the library and on the desired output and data quality. Begin optimization using a seeding concentration in the middle of the range listed in Table 54 or provided by Illumina. Follow Illumina's recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

Table 54 Illumina Kit Configuration Selection Guidelines

Instrument	Run Type	Read Length	SBS Kit Configuration	Chemistry	Seeding Concentration
MiSeq	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	9–10 pM
			600 Cycle Kit	v3	12–16 pM
iSeq 100	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2	50–150 pM
NextSeq 500/550	All Runs	2 × 100 bp or 2 × 150 bp	300 Cycle Kit	v2.5	1.2–1.5 pM
NextSeq 1000/2000	All Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	Standard SBS	650–1000 pM
				XLEAP-SBS	650–1000 pM
NovaSeq 6000	Standard Workflow Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1.5	300–600 pM
NovaSeq 6000	Xp Workflow Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1.5	200–400 pM
NovaSeq X	All Runs	2 × 100 bp or 2 × 150 bp	200 or 300 Cycle Kit	v1	90–180 pM

Set up the sequencing run to generate Read 1 and Read 2 FASTQ files for each sample using the instrument's software in standalone mode or using an Illumina run management tool such as Local Run Manager (LRM), Illumina Experiment Manager (IEM) or BaseSpace. Enter the appropriate Cycles or Read Length value for your library read length and using 8-bp dual index reads. See Table 55 showing example settings for 2 × 150 bp sequencing.

Table 55 Run settings for 2x150 bp sequencing

Run Segment	Cycles/Read Length
Read 1	151*
Index 1 (i7)	8
Index 2 (i5)	8
Read 2	151*

* Follow Illumina's recommendation to add one (1) extra cycle to the desired read length.

Follow Illumina's instructions for each platform and setup software option, incorporating the additional setup guidelines below:

- Each of the sample-level indexes (i7 and i5) requires an 8-bp index read. See "Online resource for Avida index sequences" on page 14 for information on downloading the Avida index sequences spreadsheet.
- No custom primers are used for Avida library sequencing. Leave all Custom Primers options for Read 1, Read 2, Index 1 and Index 2 primers cleared/deselected during run setup.
- Turn off any adaptor trimming tools included in Illumina's run setup and read processing software applications. Adaptors are trimmed in later processing steps to ensure proper processing of the adaptors, including the UMIs in the adaptor sequences.
- For runs set up using Illumina's LRM, IEM, or BaseSpace applications, refer to Illumina's instructions and support resources for setting up runs with custom library prep kits and index kits in the selected software. For use in these applications, the Avida index sequences provided online should be converted to .tsv/.csv file format or copied to a Sample Sheet according to Illumina's specifications for each application. If you need assistance with Avida run setup in your selected application (e.g., generating index files or Sample Sheet templates), contact the Agilent Technical Support team (see page 2) or your local representative.

Step 3. Process and analyze the reads

Typical sequencing data processing steps are outlined below. For more detailed guidance on analysis of Avida DNA sequencing data using open-source software packages, see the Avida targeted DNA sequencing technical guide (publication number G9409-90001) available at www.agilent.com.

1 Generate demultiplexed FASTQ files for each sample using Illumina's bcl2fastq, BCL Convert, or a similar software application. This process generates paired-end reads based on the dual indexes and removes sequences with incorrectly paired P5 and P7 indexes. Do not use the UMI trimming options offered in Illumina's demultiplexing software.

NOTE

If your sequence analysis pipeline excludes UMIs, you can remove the first 5 bases from Read 1 and Read 2 by masking or trimming before proceeding to downstream analysis.

If demultiplexing using bcl2fastq, UMIs may be masked by including the base mask **N5Y*,I8,I8,N5Y*** (where * is replaced with the remaining read length after subtracting the 5 masked bases, e.g., use **N5Y146,I8,I8,N5Y146** for 2x150 NGS set up as shown in Table 55 on page 80). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

If demultiplexing using BCL Convert, UMIs may be trimmed by including the following string in the sample sheet header: **OverrideCycles,N5Y*;I8;I8;N5Y*** (where * is replaced with read length after trimming, e.g., use **N5Y146;I8;I8;N5Y146** for 2x150 NGS set up as shown in Table 55 on page 80). The sum of the values following N and Y must match the read length value in the RunInfo.xml file.

Alternatively, the first 5 bases may be trimmed from the demultiplexed FASTQ files using a suitable processing tool (e.g., fgbio).

2 Use a suitable processing tool of your choice to trim and collect inline UMIs from each sequencing read. For example, UMI processing and sequence read alignment steps could be conducted with the fgbio best practice consensus pipeline, as described in: https://github.com/fulcrumgenomics/fgbio/blob/main/docs/best-practice-consensus-pipeline.md.
The output includes deduplicated reads in BAM format with, 1) single-stranded UMI consensus reads, and 2) double-stranded UMI consensus reads.

- Inline UMIs are added to both ends of the DNA inserts in the assay. To collect the UMIs and process them for analysis, one approach is to trim off 5 bases at the beginning of each read, take the first 3 of the 5 bases as UMI, and discard the remaining 2.

3 Perform variant calling and filtering using the BAM files generated in step 2 above.

4 Collect QC metrics on alignment.

5 (Optional) Generate a report containing the QC metrics and variant calls.

9 Reference

This section contains reference information, including Reagent Kit contents, index primer pair information, and troubleshooting tips.

Reagent Kit Contents

Table 1 on page 13 lists the Avida DNA reagent kits and their sub-kit boxes. Detailed contents of those sub-kit boxes are shown in Table 56 through Table 58.

Avida Sub-Kit Details

Table 56 Avida DNA Reagent Box 1

Kit Component	96 Reaction Kit (p/n 5282-0140)
End Prep Buffer	tube with purple cap
End Prep Enzyme	tube with blue cap
Ligation Buffer	bottle
Ligation Enzyme	tube with yellow cap
Adaptor for ILM	tube with orange cap
Hyb Blocker	tube with red cap
Hyb Buffer	bottle
Hyb Enhancer	tube with green cap
2X Amplification Mastermix	bottle

Table 57 Avida DNA and Duo Reagent Box 2

Kit Component	96 Reaction Kit (p/n 5282-0142)
Library Wash Buffer	bottle
Hyb Wash Buffer 1	bottle
Hyb Wash Buffer 2	bottle
Resuspension Buffer	bottle
Nuclease-Free Water	bottle

Table 58 Avida Beads Box

Kit Component	96 Reaction Kit (p/n 5282-0144)
Library Binding Beads	bottle
Capture Beads	tube with amber cap

Avida Index Primer Pair Information

The Avida Index Primer Pairs are provided pre-combined in the well of a plate (see Table 59). Each member of the primer pair contains a unique 8-bp P5 or P7 index, resulting in dual-indexed NGS libraries. One primer pair (forward and reverse primers) is provided as a single-use aliquot in each well of a 96-well plate.

See "Online resource for Avida index sequences" on page 14 for information on downloading the Avida index sequences spreadsheet.

Table 59 Avida Index Primer Pairs for ILM Content

Kit Component	96 Reaction Kit Format
Avida Index Primer Pairs for ILM	Clear 96-well plate with index pairs 1–96 - - OR - - Blue 96-well plate with index pairs 97–192

Index Primer Pair Plate Maps

Table 60 and Table 61 show the plate positions of the Avida Index Primer Pairs provided with 96 reaction kits.

Table 60 Plate map for Avida Index Primer Pairs 1-96, provided in clear plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	93
F	6	14	22	30	38	46	54	62	70	78	86	94
G	7	15	23	31	39	47	55	63	71	79	87	95
H	8	16	24	32	40	48	56	64	72	80	88	96

Table 61 Plate map for Avida Index Primer Pairs 97-192, provided in blue plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	97	105	113	121	129	137	145	153	161	169	177	185
B	98	106	114	122	130	138	146	154	162	170	178	186
C	99	107	115	123	131	139	147	155	163	171	179	187
D	100	108	116	124	132	140	148	156	164	172	180	188
E	101	109	117	125	133	141	149	157	165	173	181	189
F	102	110	118	126	134	142	150	158	166	174	182	190
G	103	111	119	127	135	143	151	159	167	175	183	191
H	104	112	120	128	136	144	152	160	168	176	184	192

Avida Custom AddOn Panels

When including a custom Avida DNA AddOn panel in the hybridization, make the protocol adjustments described below.

CAUTION Make sure that the Custom AddOn panel is compatible with the Avida base panel. When creating a custom Avida AddOn design on the SureDesign website, SureDesign verifies compatibility between the AddOn panel and the designated base panel. If needed, check with Agilent Technical Support or your local sales representative for design ID compatibility. AddOn panels are limited to 25 kb.

During preparation of the Hyb master mix on page 38, substitute the reagents and volumes in Table 20 or Table 21 with those shown in Table 62 or Table 63 below (depending on the total probes size of both the base and AddOn panels).

Table 62 Preparation of Hyb master mix using Avida DNA Custom AddOn Panel – Total probes size <500 kb

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 (bottle)	13.5 µL	172 µL	300 µL	428 µL	554 µL	810 µL	1570 µL
Hyb Blocker (red cap)	2.5 µL	32 µL	56 µL	80 µL	102 µL	150 µL	290 µL
Hyb Enhancer (amber tube with green cap)	6 µL	76 µL	134 µL	190 µL	246 µL	360 µL	698 µL
Avida DNA panel (catalog)	4 µL	50 µL	90 µL	126 µL	164 µL	240 µL	466 µL
Avida DNA Custom AddOn Panel	4 µL	50 µL	90 µL	126 µL	164 µL	240 µL	466 µL
Total Volume	**30 µL**	**380 µL**	**670 µL**	**950 µL**	**1230 µL**	**1800 µL**	**3490 µL**

Table 63 Preparation of Hyb master mix using Avida DNA Custom AddOn Panel – Total probes size ≥500 kb

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 (bottle)	13.5 µL	172 µL	302 µL	430 µL	558 µL	814 µL	1578 µL
Hyb Blocker (red cap)	2.5 µL	32 µL	56 µL	80 µL	104 µL	150 µL	292 µL
Hyb Enhancer (amber tube with green cap)	5 µL	64 µL	112 µL	158 µL	206 µL	302 µL	584 µL
Avida DNA panel (catalog)	4 µL	52 µL	90 µL	126 µL	166 µL	242 µL	468 µL
Avida DNA Custom AddOn Panel	4 µL	52 µL	90 µL	126 µL	166 µL	242 µL	468 µL
Total Volume	**29 µL**	**370 µL**	**650 µL**	**920 µL**	**1200 µL**	**1750 µL**	**3390 µL**

Troubleshooting Guide

If yield of libraries is lower than previous runs

- Make sure that the Library Binding Beads (used in "Step 7. Prepare the Library Prep master mix reagent source plate" on page 39) have equilibrated to room temperature before use.
- During "Step 6. Perform PCR cycling" on page 62, the number of cycles used in for Amplification stage 2 may require optimization. Refer to the *Note* on page 62 for guidelines.

If Library Binding Beads settle while sitting in the Library Prep master mix reagent source plate

- Before setting up the plate, allow the stock bottle of Library Binding Beads to equilibrate to room temperature and make sure the bottle is thoroughly vortexed.
- Avoid leaving the Library Prep master mix reagent source plate on ice for longer than necessary before starting the Library Prep runset.

If any of the reagents in the Library Prep master mix reagent source plate run out

- Make sure that the correct reagent volumes are added to the appropriate wells of the plate. Refer to Table 22 on page 39 and Figure 3 on page 40.
- Briefly centrifuge the plate at 50 × g for 1 second to drive the well contents off the walls.

If peak positions in the electropherogram are not as expected

- The indexing PCR reactions may have been contaminated with amplicon from another assay. Maintain separate work areas for pre-PCR and post-PCR steps. If maintaining separate areas is not an option, clean the at-risk surfaces with 10% bleach. Review "Important practices for preventing contamination" on page 11.

If low uniformity of coverage with high AT-dropout is observed in library sequencing results

- Make sure that the hybridization temperature used in the hybridization thermal cycle program is 60°C as directed in Table 26 on page 45.

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In This Book

This guide contains information to run the Avida DNA workflow using automation protocols provided with the Agilent NGS Workstation Option B.

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