Avida DNA Reagent Kits

For targeted sequencing on the Illumina platform

Library preparation, target capture, and indexing

Protocol

Version B0, June 2024

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

Notices

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In this Guide...

This guide provides an optimized protocol for preparation of target-enriched Illumina paired-end sequencing libraries using Avida DNA reagent kits.

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 gDNA Fragmentation

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

3 PCR-Free Library Preparation

This chapter describes the steps for the PCR-free library preparation section of the Avida DNA workflow. NGS libraries are prepared using adaptors that include Unique Molecular Identifiers (UMIs).

4 Target Capture

This chapter describes the steps for the target capture section of the Avida DNA workflow. The adaptor-ligated libraries are hybridized with a target-specific panel. After hybridization, the targeted molecules are captured on Capture Beads coated with Streptavidin.

5 Indexing PCR, Library Purification, and Quality Assessment

This chapter describes the steps for the indexing PCR, library purification, and quality assessment section of the Avida DNA workflow. The captured libraries are amplified and indexed, and the indexed libraries are then purified with AMPure XP Beads. Finally, the purified, indexed libraries are quantified and analyzed for quality.

6 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.

7 Reference

This section contains reference information, including Reagent Kit contents, index primer pair information, troubleshooting tips, and a quick-reference protocol for experienced users.

What's New in Version B0

- Support for new Avida DNA Discovery Cancer Panel (see Table 3 on page 12 for part number).
- Expanded information on compatible reagent kits for the Agilent Fragment Analyzer system, including addition of electropherogram images in Figure 4 and Figure 5.
- In Quick Reference Protocol, clarified storage conditions for the Avida panels and the final sequencing libraries.
- In Table 16 on page 27, corrected storage temperature of Hyb Mix 1. Added Note (page 25) on preparing aliquots of Hyb Mix 1.
- Updated thawing conditions for Adaptor for ILM (page 20 and page 22).
- In Table 32 on page 47, corrected part numbers for the Avida Bead Box kit components.
- Updated the instructions for "Step 2. Capture Bead Preparation" on page 30, including adding details based on number of reactions.
- Updated storage conditions for the Stopping Point on page 37.
- Expanded recommended Qubit Assay Kits to include Qubit dsDNA HS and BR Assay Kits.
- In the Quick Reference Protocol, added link to the new graphics- based Avida DNA Quick Start Protocol.

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Before You Begin

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

NOTE Agilent guarantees performance and provides technical support for the 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.

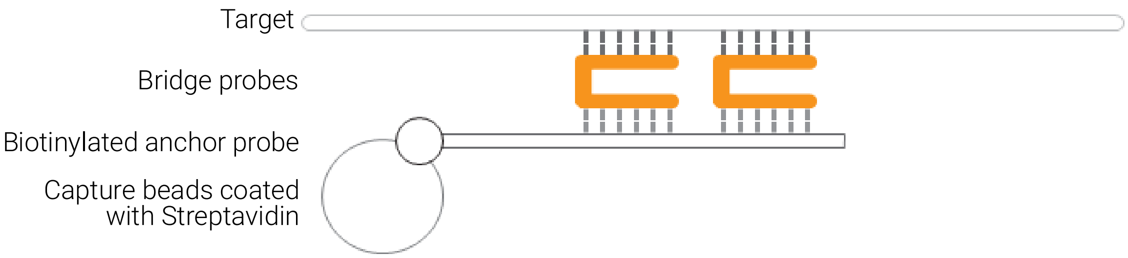

Figure 1 Hybridization of target to biotinylated anchor probe mediated by bridge probes

Overview of the 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 14 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 library complexity which enables highly sensitive detection of genomic changes.

The workflow has three sections (see Table 1). You can expect the entire process to take approximately 5 hours to complete. The resulting libraries are ready for next-generation sequencing (NGS) on Illumina platforms.

Table 1 Workflow sections 1–3 and estimated time requirements for up to 8 samples

No.	Section	Description	Time
1	PCR-free library prep	The PCR-free library prep converts cfDNA or fragmented gDNA into adaptor-ligated molecules by end-repair and adaptor ligation	2 hours
2	Target capture	Target capture enriches specific targets from PCR-free libraries by probe hybridization and washes	1.5 hours
3	Indexing PCR and purification	Indexing PCR incorporates sample indexes and P5/P7 sequences necessary for Illumina sequencing	1.5 hours

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 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 (BSL1) safety rules.

Procedural notes

- This protocol has been developed using PCR 8-well strip tubes with individually attached caps or with tube cap strips. If using tube cap strips, for each protocol step that requires removal of tube cap strips, reseal the tubes with a fresh strip of caps. Cap deformation may result from exposure of the cap strips to the heated lid of the thermal cycler and from other procedural steps. Reuse of strip caps can cause sample loss, sample contamination, or imprecision in sample temperatures during thermal cycler incubation steps.
- If handling multiple samples during washes (in Chapter 4, “Target Capture”):

1. Pre-aliquot reagents to a pipetting reservoir of appropriate size.
2. Use a multichannel pipette for washes.

- **Because the Avida DNA workflow does not include a pre-capture amplification step, make sure no residual sample is left in the tube or pipette tip during sample transferring and pipetting to prevent any sample loss.**
- At certain steps that use magnetic beads, the complete removal of all supernatant is critical. The recommended technique for collecting residual supernatant at the bottom of the tubes depends on the magnet.
  - For magnets that collect beads on tube sides: Tap the magnet stand on the bench 5 times.
  - For magnets that collect beads at tube bottoms: Remove tubes from magnet and briefly spin. Return tubes to magnet before removing liquid.
- Reagents requiring –20°C storage are temperature sensitive and appropriate care should be taken during storage and handling. Avoid repeated freeze-thaw cycles. Make sure all reagents previously stored at –20°C are completely thawed before use.
- To process multiple samples, the protocol includes steps for preparation of reagent mixtures with overage, which are afterward distributed to the DNA library samples. To make reagent mixtures, scale reagents' volumes based on the number of reactions, using 10% excess volume to compensate for any pipetting loss. Add reagents in the order listed when preparing mixtures, then vortex to mix and briefly spin. Mixtures for preparation of 8 or 16 samples are shown in tables as examples.
- 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 2. lists the Avida DNA reagent kits and the sub-kit boxes provided with each kit. The 16-reaction kits provide sufficient reagents for 2 assays with 8 samples. The 96-reaction kits provide sufficient reagents for 6 assays with 16 samples. The components of the sub-kit boxes are provided in Chapter 7, “Reagent Kit Contents,” starting on page 47.

Table 3 lists the Avida panels compatible with this Protocol.

Table 2 Avida DNA reagent kits

Avida DNA reagent kits	Sub-kit boxes	Storage Temperature
P/N G9409A Avida DNA reagent kit with 1-16 index primer pairs for ILM, 16 reactions	Avida DNA Reagent Box 1, 16 Reactions	−20°C
	Avida DNA and Duo Reagent Box 2, 16 Reactions	Room temperature
	Avida Beads Box, 16 Reactions	4°C
	Avida Index Primer Pairs 1–16 for ILM	−20°C
P/N G9409B Avida DNA reagent kit with 17-32 index primer pairs for ILM, 16 reactions	Avida DNA Reagent Box 1, 16 Reactions	−20°C
	Avida DNA and Duo Reagent Box 2, 16 Reactions	Room temperature
	Avida Beads Box, 16 Reactions	4°C
	Avida Index Primer Pairs 17–32 for ILM	−20°C
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 3 Compatible Avida panels

Capture Probes Panel	Size	Design ID	P/N for 16-reactions	P/N for 96-reactions
Avida DNA Discovery Cancer Panel	2.68 Mb	D3496941	5280-0040	5280-0044
Avida DNA Expanded Cancer Panel	345 kb	D3483241	5280-0046	5280-0047
Avida DNA Focused Cancer Panel	26 kb	D3483231	5280-0049	5280-0050

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 4. Input quantity ranges are based on Qubit Assay quantification of the DNA samples. DNA should be stored in 1X 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 2, “gDNA Fragmentation” for instructions). Since cfDNA typically has an average size of 170 base pairs, no further fragmentation is required.

Table 4 **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 5 through Table 7 for additional reagents and equipment required to complete the workflow.

Table 5 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 dsDNA HS or BR 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 6 Required equipment

Description	Vendor and Part Number
Thermal Cycler with 96-well, 0.2 mL block	Various suppliers
PCR 8-well strip tubes, 0.2-mL, with individually attached caps or with strip caps	VWR p/n 76318-798, Genesee p/n 27-125U, or consult the thermal cycler manufacturer's recommendations
Nucleic acid analysis system (instrument and consumables)	Select one system from Table 7 on page 15
Qubit 4 Fluorometer and Qubit Assay Tubes (optional)	Thermo Fisher Scientific p/n Q33238 and p/n Q32856
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
Microcentrifuge	Eppendorf microcentrifuge, model 5417C or equivalent
Centrifuge for 8-well strip tubes, 3000–5000 rpm	General laboratory supplier
Centrifuge for 96-well plate (if using 96-reaction kits)	General laboratory supplier
DynaMag-96 Side Magnet or Magnum EX Universal 96-well Magnet Plate	Thermo Fisher Scientific p/n 12331D or Alpaqua p/n A000380
Magnet for 1.5-mL tubes (optional)	Thermo Fisher Scientific p/n 12321D or equivalent
Multichannel and single channel pipettes	Rainin Pipet-Lite Multi Pipette or equivalent
Reagent reservoirs for use with multichannel pipettes (optional)	VWR 10015-236 or equivalent
Sterile, nuclease-free aerosol barrier pipette tips, vortex mixer, cold rack, ice bucket, and powder-free gloves	General laboratory supplier

Table 7 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 8 lists information on equipment for fragmentation of gDNA samples using mechanical shearing. Sample fragmentation is required for high-quality gDNA samples and FFPE-derived gDNA samples but not for cfDNA samples.

Table 8 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

gDNA Fragmentation

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

NOTE Fragmentation is only required for gDNA samples. If you are using cfDNA samples, proceed to Chapter 3, “PCR-Free Library Preparation.”

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. 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 briefly spin. 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 9.

Table 9 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 strip tube sample 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 3, “PCR-Free Library Preparation.”

This is not a stopping point.

3

PCR-Free Library Preparation

This chapter describes the steps for the PCR-free library preparation section of the Avida DNA workflow. NGS libraries are prepared using adaptors that include Unique Molecular Identifiers (UMIs).

See Table 4 on page 14 for DNA sample input requirements.

This chapter uses the reagents listed in Table 10.

Table 10 Reagents for PCR-free library preparation

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 (tube with green cap or 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.	
Library Binding Beads (tube with white cap or bottle)	Equilibrate to room temperature for at least 15 minutes.	Avida Beads Box, stored at 4°C
Nuclease-free Water (tube with clear cap or 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

* Compatible Avida DNA Panels are listed in Table 3 on page 12.

CAUTION

Review the "Procedural and Safety Notes" on page 10 before getting started.

Because the Avida DNA workflow does not include a pre-capture amplification step, make sure no residual sample is left in the tube or pipette tip during sample transferring and pipetting to prevent any sample loss.

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 4. 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° heat block until the buffer is used in Chapter 4.
- 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° heat block until the buffer is used in Chapter 4.

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. End prep (end repair and dA-tailing)

1 Thaw the End Prep Buffer at room temperature, then thoroughly vortex. Inspect the buffer to make sure that no small particles are visible. If particles are visible, continue to vortex until they dissolve.

2 For each sample, add an appropriate quantity of DNA (based on sample type, see below) to a strip tube 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 4 on page 14 for complete information on the DNA sample input requirements. Keep DNA samples on ice.

   - cfDNA samples: 1–100 ng
   - fragmented gDNA samples: 10–100 ng (prepared in Chapter 2)

3 Prepare the appropriate volume of End Prep Mix by combining the reagents in Table 11 at room temperature. Combine the reagents in the order shown. Gently vortex the End Prep Mix at low speed, then briefly spin.

   Once prepared, keep the End Prep Mix on ice. The End Prep Mix is stable for up to 2 hours at 4°C.

Table 11 Preparation of End Prep Mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
End Prep Buffer	7 µL	63 µL	126 µL
End Prep Enzyme	3 µL	27 µL	54 µL
Total	**10 µL**	**90 µL**	**180 µL**

4 Add 10 µL of End Prep Mix to each tube containing a DNA sample. Mix by gentle vortexing or by pipetting up and down 15–20 times using a pipette set to 50 µL. Briefly spin the strip tubes then keep on ice.

   A small amount of bubbles in the end prep reactions does not affect performance.

5 Program the thermal cycler to run the end prep thermal cycler program shown in Table 12. Set the heated lid to 75°C. Load the strip tubes and run the program.

Table 12 Thermal cycler program for end prep

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	65°C	30 minutes
Step 3	4°C	Hold

6 Start thawing the Ligation Buffer and the Adaptor for ILM at room temperature in preparation for the next step. Thoroughly vortex once thawed. Keep the thawed Adaptor for ILM on ice. Inspect the Ligation Buffer to make sure that no small particles are visible. If particles are visible, continue to vortex until they dissolve.

Step 2. Ligation

1 While the end prep thermal cycler program is running, prepare the Ligation Master Mix by combining the reagents in Table 13 in a 1.5-mL tube at room temperature. Vortex the Ligation Master Mix, then briefly spin the tube. Once prepared, keep it on ice until needed.

CAUTION The Ligation Buffer and Ligation Enzyme are viscous. Take care when pipetting these reagents and mixtures containing these reagents.

The Ligation Master Mix is stable for up to 2 hours at 4°C.

Table 13 Preparation of Ligation Master Mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
Ligation Buffer	25 µL	225 µL	450 µL
Ligation Enzyme	6 µL	54 µL	108 µL
Total	**31 µL**	**279 µL**	**558 µL**

2 When the end prep thermal cycler program is complete, remove the strip tubes containing the end-repaired/dA-tailed DNA samples. Keep at room temperature.

Make sure to stop the thermal cycler program to allow the thermal block and heated lid to equilibrate to room temperature.

3 Add 5 µL of Adaptor for ILM to each DNA sample.

CAUTION Do NOT add Adaptor for ILM to the Ligation Master Mix. Avoid exposing the Adaptor to room temperature conditions.

4 Add 31 µL of Ligation Master Mix to each sample. Mix by gentle vortexing or by pipetting up and down 15–20 times using a pipette set to 70 µL. Briefly spin the samples.

5 Make sure that the thermal cycler has equilibrated to room temperature. Program the thermal cycler to run the ligation thermal cycler program shown in Table 14 with the heated lid off. Load the strip tubes and run the program.

Table 14 Thermal cycler program for ligation

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

6 During the thermal cycler program for ligation, prepare the following reagents as described below in preparation for upcoming steps.

- Equilibrate the Library Binding Beads to room temperature for at least 15 minutes before use in "Step 3. Library bead binding."
- Heat the Hyb Buffer to 37°C in a water bath or heat block for at least 10–20 minutes, or until the Hyb Buffer is completely solubilized. If the Hyb Buffer is supplied in a bottle, and the 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. Once the Hyb Buffer is completely solubilized, transfer to room temperature until use. The Hyb Buffer is used in Chapter 4, "Target Capture."

NOTE If your laboratory does not have a water bath or heat block, you can heat the tube or 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, especially if the Hyb Buffer is supplied in a bottle.

- Thaw the Hyb Blocker, Hyb Enhancer, and Avida Panel at room temperature. Transfer the Avida Panel to ice once thawed. Keep the Hyb Blocker and Hyb Enhancer at room temperature until use. The Hyb Blocker and Avida Panel are used to prepare Hyb Mix 1 in Table 15. The Hyb Enhancer is used in Chapter 4, "Target Capture."

CAUTION The Hyb Enhancer must be thawed at room temperature. If a precipitate is visible after thawing, thoroughly vortex until the solution clears.

Step 3. Library bead binding

1 Once the thermal cycler program for ligation completes, remove the strip tubes containing the adaptor-ligated DNA samples from the thermal cycler.

2 Thoroughly vortex the stock bottle or stock tube of Library Binding Beads to mix. Set the tube or bottle upright for 5 minutes to allow any particles to settle to the bottom.

3 Add 87 µL (~0.9X volume) of Library Binding Beads to each adaptor-ligated DNA sample, bringing the sample volume to 183 µL. Mix by pipetting up and down 15–20 times using a pipette set to 150 µL. Spin for 1 to 2 seconds to collect the liquid at the bottom of the tube, making sure the beads do not settle.

Pipette slowly to avoid creating excess bubbles. Bubbles can compromise library binding efficiency.

4 Incubate the library bead binding reactions for 10 minutes at room temperature.

5 **During the 10-minute incubation,** prepare the Hyb Mix 1 by combining the reagents in Table 15 in a 1.5-mL tube. Vortex the Hyb Mix 1 thoroughly, then briefly spin the tube. Set aside at room temperature until needed in the next chapter.

NOTE When handling multiple samples, Agilent recommends aliquoting 26 µL of Hyb Mix 1 into the tubes of a fresh strip tube (one tube per sample). Then, during step 1 on page 28, use a multichannel pipette to transfer 24 µL of Hyb Mix 1 from the strip tube to the beads bound with adaptor-ligated library. This technique helps prevent the beads from drying out.

Table 15 Preparation of Hyb Mix 1

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
Nuclease-Free Water	17.5 µL	157.5 µL	315 µL
Avida DNA Panel	4 µL	36 µL	72 µL
Hyb Blocker	2.5 µL	22.5 µL	45 µL
Total	**24 µL**	**216 µL**	**432 µL**

6 When the 10-minute incubation is complete, place the strip tubes on a magnet for at least 2 minutes or until the solution is clear.

NOTE The solution may take longer than 2 minutes to become clear. Visually inspect the tubes to make sure that the beads have fully aggregated and the solution is clear before proceeding.

7 Remove and discard all supernatant from each tube without removing or disturbing the beads. **Leave the strip tubes on the magnet until instructed to remove them.**

8 Add 180 µL of Library Wash Buffer to each tube without disturbing the bead pellet. Incubate for 2 minutes at room temperature.

9 Remove and discard all supernatant from each tube.

10 (Optional) Repeat step 8 through step 9 for an additional wash.

This second wash may be help improve percent on-target in sequencing results, but it may slightly reduce molecular recovery.

11 Collect all residual supernatant at the bottom of the tubes, then remove and discard the residual supernatant from each tube using a 20 µL pipette.

Refer to "Procedural notes" on page 10 for best practices on removing all traces of supernatant based on magnet type.

12 Remove the strip tubes from the magnet.

The adaptor-ligated libraries remain bound to the Library Binding Beads.

13 Proceed **immediately** to step 1 on page 28 (in Chapter 4, "Target Capture") and add the Hyb Mix 1 that you prepared earlier to make sure that the beads do not dry out.

4

Target Capture

This chapter describes the steps for the target capture section of the Avida DNA workflow. The adaptor-ligated libraries are hybridized with a target-specific panel. After hybridization, the targeted molecules are captured on Capture Beads coated with Streptavidin.

This chapter uses the reagents listed in Table 16.

Table 16 Reagents for target capture

Reagent	Usage Notes	Kit/Storage Location
Hyb Buffer (tube with clear cap or bottle)	Heat to 37°C in water bath or heat block for at least 10–20 minutes before use. Once solution becomes clear, keep at room temperature.	Avida DNA Reagent Box 1, stored at −20°C
Hyb Enhancer (amber tube with green cap)	Thaw at room temperature.	
Capture Beads (tube with amber cap)	Thoroughly vortex to mix before use.	Avida Beads Box, stored at 4°C
Nuclease-free Water (tube with clear cap or 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 (tube with red cap or bottle)	—	
Hyb Mix 1	Prepared in previous chapter (page 25) and kept at room temperature until use.	

Step 1. Hybridization

1. Add 24 µL of Hyb Mix 1 (prepared on page 25) to the beads bound with adaptor-ligated library generated in the last step of the previous chapter. Resuspend the beads by gentle vortexing or by pipetting up and down 15–20 times. Briefly spin the strip tubes.

NOTE If during preparation of Hyb Mix 1 (page 25) you distributed 26 µL of Hyb Mix 1 into the tubes of a fresh strip tube, you can now use a multichannel pipette to transfer 24 µL from the strip tube to the beads bound with adaptor-ligated library.

2. Thoroughly vortex the Hyb Buffer that has been kept at 37°C.
3. Prepare the Hyb Mix 2 based on the Avida DNA panel size.
   - Table 17 - panels <500 kb (Focused Cancer Panel and Expanded Cancer Panel)
   - Table 18 - panels ≥500 kb (Discovery Cancer Panel)

   Combine the reagents listed in the appropriate table in a 1.5-mL tube. Vortex the Hyb Mix 2 thoroughly, then briefly spin the tube. Keep at room temperature.

   Keep the Hyb Buffer at room temperature until later use.

Table 17 Preparation of Hyb Mix 2 for panels <500 kb

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
Hyb Buffer	30 µL	270 µL	540 µL
Hyb Enhancer	6 µL	54 µL	108 µL
Total	**36 µL**	**324 µL**	**648 µL**

Table 18 Preparation of Hyb Mix 2 for panels ≥500 kb

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
Hyb Buffer	31 µL	279 µL	558 µL
Hyb Enhancer	5 µL	45 µL	90 µL
Total	**36 µL**	**324 µL**	**648 µL**

4. Add 36 µL of Hyb Mix 2 to each bead mix containing Hyb Mix 1 (from step 1 above), bringing the total volume of each hybridization reaction to 60 µL.
5. Mix by pipetting up and down 15–20 times using a pipette set to 50 µL. Spin for 1 to 2 seconds to collect the liquid at the bottom of the tube, making sure the beads do not settle.

6 Program the thermal cycler to run the hybridization thermal cycler program shown in Table 19. Set the heated lid to 103°C. Load the strip tubes and run the program.

Table 19 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 19 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.

Step 2. Capture Bead Preparation

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

Capture bead preparation can be performed during the last 15 minutes of the hybridization thermal cycler program.

1 Thoroughly vortex the Hyb Buffer that has been kept at room temperature since its last use in "Step 1. Hybridization."

2 Thoroughly pipette or vortex the Capture Beads stock to make sure the solution is homogeneous.

3 8 µL of Capture Beads are required per reaction. Calculate the total volume of beads needed for all the samples, including 10% overage, and transfer that volume into a PCR tube or 1.5-mL tube.

NOTE Preparing the Capture Beads in a 1.5-mL tube requires a magnet that accommodates that tube size. If needed, the required volume of Capture Beads can be divided among multiple PCR tubes.

4 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.

5 Add the appropriate volume of the preheated Hyb Wash Buffer 1 to the Capture Beads using the guidelines in Table 20. 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.

Table 20 Wash volumes of Hyb Wash Buffer 1 based on number of hybridization reactions

Number of reactions	Volume of Hyb Wash Buffer 1
1–16	100 µL
17–32	200 µL
33–48	300 µL
49–64	400 µL
65–80	500 µL
81–96	600 µL

6 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.

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

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

8 Remove the tube of Capture Beads from the magnet. Resuspend the beads in Hyb Buffer at the same volume you originally started with (as calculated in step 3, above). Mix by pipetting up and down 15–20 times (avoid creating excess bubbles).

NOTE Make sure to use Hyb Buffer to resuspend the Capture Beads.

9 Add 8 µL of resuspended Capture Beads to each tube of a fresh strip tube (one for each hybridization reaction).

Step 3. Bead Capture

1 Once the hybridization thermal cycler program completes, remove the strip tubes containing the hybridization reactions from the thermal cycler and set them aside. Stop the hybridization thermal cycler program, and start the washing thermal cycler program shown in Table 21. Set the heated lid to 75°C.

Table 21 Thermal cycler program for washing

Step	Temperature	Time
Step 1	60°C	Hold

2 Briefly spin the strip tubes then place on a magnet. Keep the tubes on the magnet for 30 seconds or until the solution becomes clear (up to 1 minute).

3 **Carefully transfer the supernatant from each tube** (making sure to get all the supernatant) to a tube containing an 8-µL aliquot of prepared Capture Beads (from step 9 on page 31). Mix by pipetting up and down 15–20 times or until the beads are fully mixed (avoid vortexing at this step). Spin for 1 to 2 seconds to collect the liquid at the bottom of the tubes, making sure the beads do not settle.

4 Perform bead binding by placing the strip tubes on the thermal cycler running at 60°C for 10 minutes.

5 After 10 minutes, remove the strip tubes from the thermal cycler and place on a magnet for 30 seconds or until the solution becomes clear (up to 1 minute). Leave the thermal cycler running at 60°C. Remove and discard all supernatant.

6 Remove the strip tubes from the magnet and immediately proceed to “Step 4. Washing.”.

Step 4. Washing

When handling multiple samples, Agilent recommends transferring Hyb Wash Buffer 1, Hyb Wash Buffer 2, and Resuspension Buffer into reagent reservoirs and pipetting the buffers using a multichannel pipette.

Refer to "Procedural notes" on page 10 for best practices on removing all traces of supernatant based on magnet type.

CAUTION For step 1 through step 5 in this section, do NOT vortex or spin the samples. Carefully handle the tubes to prevent any splashing.

1 Add 150 µL of preheated Hyb Wash Buffer 1 to each tube containing the Capture Beads/library DNA hybrids. Mix by pipetting up and down at least 15–20 times, making sure the beads are fully resuspended.

2 Put the strip tubes on a magnet for 30 seconds or until the solution becomes clear. Remove and discard the supernatant from each tube. Use a 20 µL pipette to remove all residual Hyb Wash Buffer 1.

3 Remove the strip tubes from the magnet. Add 100 µL of Hyb Wash Buffer 1 to each tube. Mix by pipetting up and down at least 15–20 times, making sure the beads are fully resuspended. **Then, transfer the entire mix (beads, buffer, and any bubbles that may have formed) to a fresh strip tube**.

4 Place the strip tubes on the thermal cycler running at 60°C for 3 minutes.

5 After 3 minutes, remove the strip tubes from the thermal cycler and stop the thermal cycler wash program.

6 Place the strip tubes on the magnet for 30 seconds or until the solution becomes clear. Remove and discard the supernatant from each tube. Use a 20 µL pipette to remove all residual Hyb Wash Buffer 1.

7 Remove the strip tubes from the magnet.

8 Add 150 µL of room temperature Hyb Wash Buffer 2 to each tube. Mix by pipetting up and down at least 15–20 times, making sure the beads are fully resuspended. Briefly spin.

Stopping Point If you do not continue to the next step, store the samples in Hyb Wash Buffer 2 at 4°C overnight.

9 Place the strip tubes on the magnet for 1 minute or until the solution becomes clear. Remove and discard the supernatant from each tube. Use a 20 µL pipette to remove all residual Hyb Wash Buffer 2.

10 Remove the strip tubes from the magnet.

11 Add 20 µL of Resuspension Buffer to each tube. Resuspend the beads by gently vortexing at low speed or by flicking the tubes. Briefly spin. Proceed to Chapter 5, "Indexing PCR, Library Purification, and Quality Assessment.".

CAUTION Do not discard the beads. The entire 20 µL of resuspended beads with captured DNA will go into indexing PCR.

5

Indexing PCR, Library Purification, and Quality Assessment

This chapter describes the steps for the indexing PCR, library purification, and quality assessment section of the Avida DNA workflow. The captured libraries are amplified and indexed, and the indexed libraries are then purified with AMPure XP Beads. Finally, the purified, indexed libraries are quantified and analyzed for quality.

This chapter uses the reagents listed in Table 22.

Table 22 Reagents for indexing PCR, library purification, and quality assessment

Reagent	Usage Notes	Kit/Storage Location
2X Amplification Mastermix (tube with white cap or bottle)	Thaw on ice.	Avida DNA Reagent Box 1, stored at −20°C
Avida Index Primer Pairs	Primer pairs are provided in either a strip or a plate. Thaw strip or plate on ice.	Stored at −20°C
Nuclease-free Water (tube with clear cap or bottle)	—	Avida DNA and Duo Reagent Box 2, stored at Room Temperature
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

Step 1. Indexing PCR

CAUTION Take precautions to avoid amplicon contamination during setup of the indexing PCR reactions. Review “Important practices for preventing contamination” on page 10.

---

1. Thaw the 2X Amplification Mastermix and Avida Index Primer Pairs on ice before use. Once thawed, gently vortex the 2X Amplification Mastermix at low speed, and briefly centrifuge the strip or plate of Avida Index Primer Pairs.
2. To each tube containing the resuspended Capture Beads with captured DNA (from step 11 on page 33), add 25 µL of 2X Amplification Mastermix to each tube. Mix by pipetting up and down 15–20 times. Briefly spin.
3. Add 5 µL of the appropriate Avida Index Primer Pair to each tube (use a pipette tip to pierce the seal of the tube or well containing the primer pair). Mix by pipetting up and down 15–20 times. Briefly spin.

   Use a different indexing primer pair for each sample to be sequenced in the same lane.

   The tubes now contain all reagents needed for indexing PCR. Table 23 summarizes the contents in each PCR reaction.

Table 23 Indexing PCR reaction components

Reagent	Volume per PCR reaction
Resuspended beads with captured DNA (from step 11 on page 33)	20 µL
2X Amplification Mastermix	25 µL
Avida Index Primer Pair	5 µL
Total	**50 µL**

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.

---

4. Program the thermal cycler to run the indexing PCR thermal cycler program shown in Table 24. Set the heated lid to 103°C. Load the strip tubes and run the program.

Table 24 Thermal cycler program for 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 25 for recommendations	98°C	10 seconds
		72°C	1 minute
Final extension	1	72°C	1 minute
Final hold	1	4°C	Hold

Table 25 Indexing PCR cycle number recommendations for Amplification stage 2

Panel size	Number of cycles	Notes
>100 kb	16 or fewer	For the Avida DNA Discovery Cancer Panel: 11 cycles For the Avida DNA Expanded Cancer Panel: 14 cycles
>50 kb to 100 kb	17	—
10 kb to 50 kb	18	Includes the Avida DNA Focused Panel
<10 kb	19	—

NOTE

The cycle number recommendations in Table 25 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.

5 Once the thermal cycler program for indexing PCR completes, remove the strip tubes containing the amplified, indexed libraries from the thermal cycler. Put on ice.

Stopping Point If you do not continue to the next step, store the indexed libraries at 4°C overnight or at –20°C for up to 72 hours. Avoid prolonged storage >72 hours.

Step 2. Library Purification

1. Equilibrate the AMPure XP Beads at room temperature for 30 minutes before use.
2. Prepare 400 µL of fresh 80% ethanol (dilute 100% ethanol with Nuclease-free Water) per library, multiplied by the number of libraries plus a 10% overage.
3. Thoroughly vortex the stock of AMpure XP Beads to mix. Add 50 µL (1X volume) of the AMPure XP Beads to each tube containing an amplified, indexed library. Mix by pipetting up and down 15–20 times.

NOTE There is no need to remove the Capture Beads from the library samples prior to addition of the AMPure XP Beads.

4. Incubate for 5 minutes at room temperature.
5. Place the strip tubes on a magnet for 2 minutes or until the solution is clear. Remove and discard the supernatant from each tube.
6. With the tubes still on the magnet, add 200 µL of freshly prepared 80% ethanol to each tube without disturbing the beads. Incubate for 30 seconds, then carefully remove and discard the supernatant from each tube.
7. Perform an additional 80% ethanol wash by repeating step 6 for a total of 2 washes.
8. Use a 20 µL pipette to remove all residual 80% ethanol. Air dry the beads for up to 3 minutes, keeping the tubes on the magnet with the lids off. Do not over-dry the beads.
9. Remove the strip tubes from the magnet, then add 23 µL of 1X Low TE Buffer to each tube. Mix by pipetting up and down 15–20 times. Briefly spin.
10. Incubate at room temperature for 2 minutes.
11. Place the strip tubes on a magnet for 2 minutes or until the solution becomes clear.
12. **Transfer 20 µL of the eluate from each tube to a new tube**, being careful to avoid bead carryover.

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

Step 3. Library Quality Assessment and Quantification

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. Analyze a sample of each library using one of the platforms listed in Table 26. Follow the instructions in the linked user guide provided for each assay.

Table 26 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	One of the following: 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.

Each analysis method provides an electropherogram showing the size distribution of fragments in the sample and tools for determining the concentration of DNA in the sample. See Table 27 for fragment size distribution guidelines.

To illustrate typical results, representative electropherograms generated using the TapeStation system are provided in Figure 2 (cfDNA sample) and Figure 3 (gDNA sample). Representative electropherograms generated on the Fragment Analyzer system are provided in Figure 4 (cfDNA sample) and Figure 5 (gDNA sample).

Determine the concentration of the library DNA by integrating under the entire peak.

Table 27 Library qualification guidelines

Input DNA type	Expected DNA fragment size peak position
cfDNA	320 bp (see Figure 2 and Figure 4 for sample electropherograms)
Fragmented gDNA[*]	300–450 bp (see Figure 3 and Figure 5 for sample electropherograms)

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

Stopping Point If you do not sequence the libraries immediately, store the tubes at 4°C overnight or at –20°C for prolonged storage.

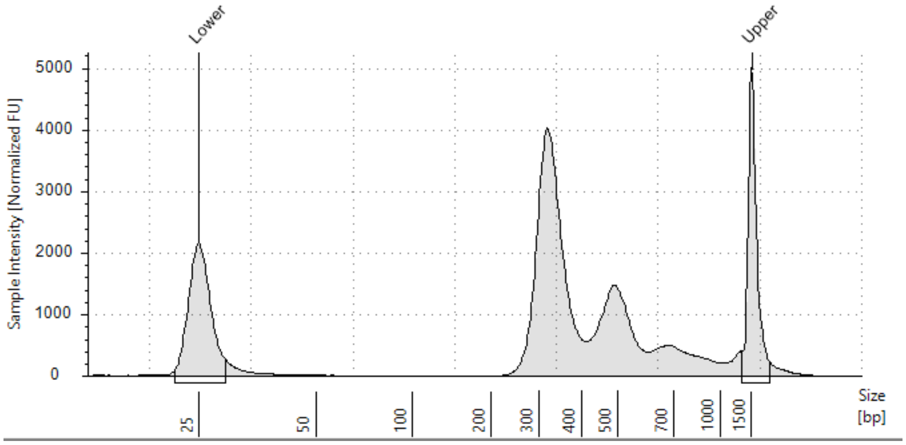

Figure 2 Library prepared from 10 ng of a cfDNA sample hybridized with the Avida DNA Focused Cancer Panel and analyzed using a D1000 ScreenTape assay on the TapeStation.

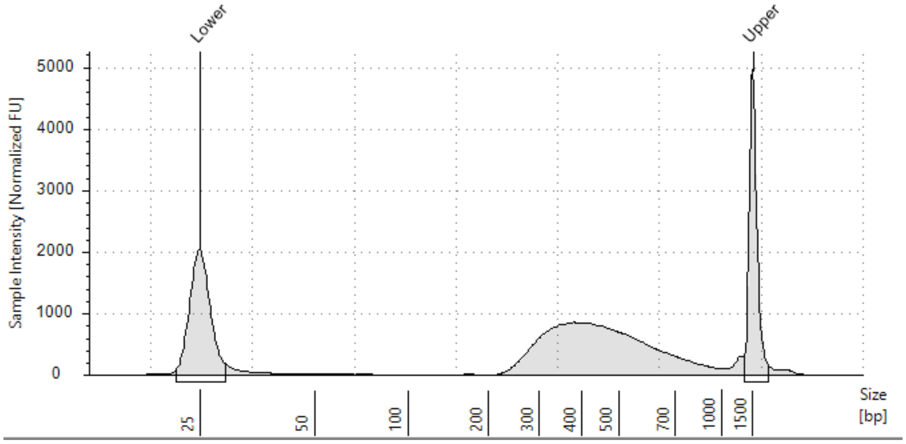

Figure 3 Library prepared from 10 ng of a high-quality gDNA sample hybridized with the Avida DNA Focused Cancer Panel and analyzed using a D1000 ScreenTape assay on the TapeStation.

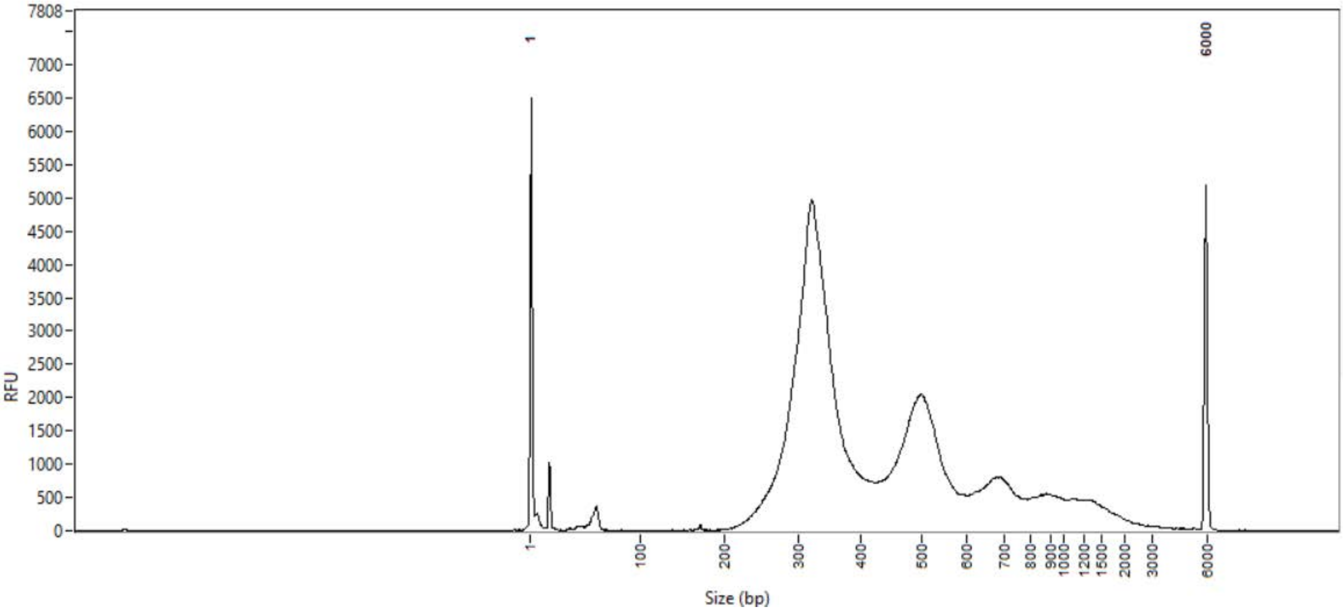

Figure 4 Library prepared from 10 ng of a cfDNA sample hybridized with the Avida DNA Focused Cancer Panel and analyzed using the NGS Fragment Kit on the Fragment Analyzer.

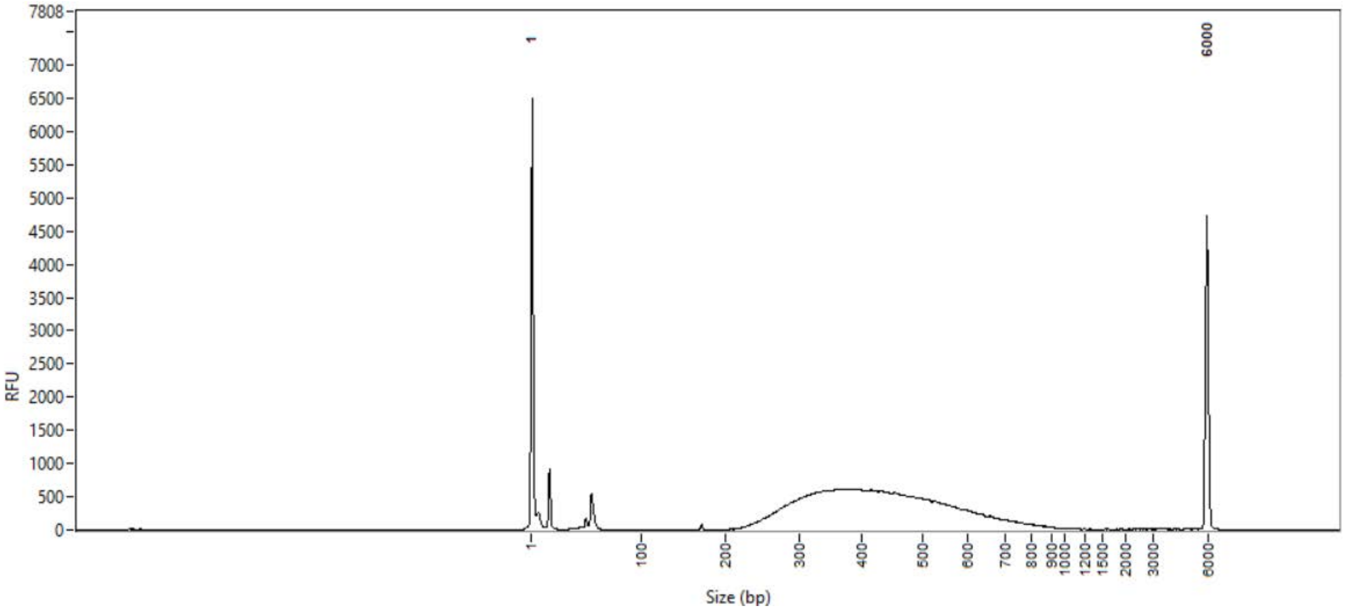

Figure 5 Library prepared from 10 ng of a high-quality gDNA sample hybridized with the Avida DNA Focused Cancer Panel and analyzed using the NGS Fragment Kit on the Fragment Analyzer.

6

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. Pool samples for multiplexed sequencing

The number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the sequencer used, together with the amount of sequencing data required per sample.

Combine the libraries such that each indexed library is present in equimolar amounts in the pool using one of the following methods. Use the diluent specified by your sequencing provider, such as 1X Low TE Buffer, for the dilution steps.

Method 1: Dilute each library sample to be pooled to the same final concentration (typically 4–15 nM, or the concentration of the most dilute sample) then combine equal volumes of all samples to create the final pool.

Method 2: Starting with library samples at different concentrations, add the appropriate volume of each sample to achieve equimolar concentration in the pool, then adjust the pool to the desired final volume using 1X Low TE Buffer. The formula below is provided for determination of the amount of each indexed sample to add to the pool.

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

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

C(f) is the desired final concentration of all the DNA in the pool (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 28 shows an example of the amount of 4 index-tagged samples (of different concentrations) and 1X Low TE Buffer needed for a final volume of 20 µL at 10 nM DNA.

Table 28 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
1X Low TE Buffer					7.6

Step 2. 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 6).

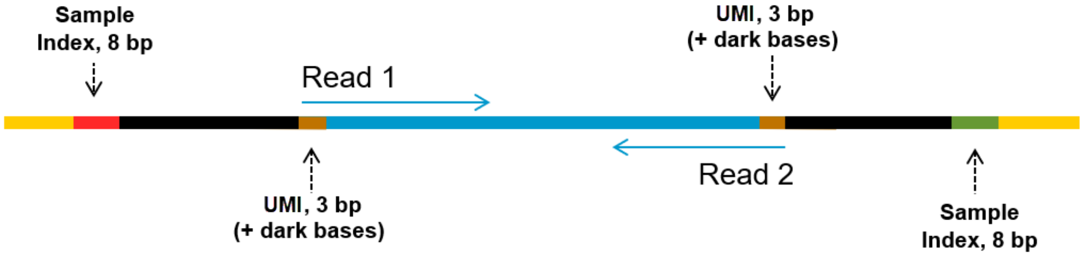

Figure 6 Content of Avida sequencing library. Each fragment contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), unique dual sample indexes (red and green), duplex UMIs (brown), and the library PCR primers (yellow).

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 3. Sequence the libraries

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 29 showing example settings for 2 × 150 bp sequencing.

Table 29 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 13 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 4. Process and analyze the reads

Typical sequencing data processing steps are outlined below.

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 29 on page 44). 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 29 on page 44). 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.

7

Reference

This section contains reference information, including Reagent Kit contents, index primer pair information, troubleshooting tips, and a quick-reference protocol for experienced users.

Reagent Kit Contents

Table 2 on page 12 lists the Avida DNA reagent kits and their sub-kit boxes. Detailed contents of those sub-kit boxes are shown in Table 30 through Table 32.

Avida Sub-Kit Details

Table 30 Avida DNA Reagent Box 1

Kit Component	16 Reaction Kit (p/n 5282-0139)	96 Reaction Kit (p/n 5282-0140)
End Prep Buffer	tube with purple cap	tube with purple cap
End Prep Enzyme	tube with blue cap	tube with blue cap
Ligation Buffer	tube with green cap	bottle
Ligation Enzyme	tube with yellow cap	tube with yellow cap
Adaptor for ILM	tube with orange cap	tube with orange cap
Hyb Blocker	tube with red cap	tube with red cap
Hyb Buffer	tube with clear cap	bottle
Hyb Enhancer	amber tube with green cap	amber tube with green cap
2X Amplification Mastermix	tube with white cap	bottle

Table 31 Avida DNA and Duo Reagent Box 2

Kit Component	16 Reaction Kit (p/n 5282-0141)	96 Reaction Kit (p/n 5282-0142)
Library Wash Buffer	bottle	bottle
Hyb Wash Buffer 1	bottle	bottle
Hyb Wash Buffer 2	bottle	bottle
Resuspension Buffer	tube with red cap	bottle
Nuclease-Free Water	tube with clear cap	bottle

Table 32 Avida Beads Box

Kit Component	16 Reaction Kit (p/n 5282-0143)	96 Reaction Kit (p/n 5282-0144)
Library Binding Beads	tube with white cap	bottle
Capture Beads	tube with amber cap	tube with amber cap

Avida Index Primer Pair Information

The Avida Index Primer Pairs are provided pre-combined in the well of either a strip tube or a plate (see Table 33). 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 an 8-well strip tubes or 96-well plate.

See “Online resource for Avida index sequences” on page 13 for information on downloading the Avida index sequences spreadsheet.

Table 33 Avida Index Primer Pairs for ILM Content

Kit Component	16 Reaction Kit Format	96 Reaction Kit Format
Avida Index Primer Pairs for ILM	Black 8-well strip tube with index pairs 1-8, AND Blue 8-well strip tube with index pairs 9-16 - - OR - - Red 8-well strip tube with index pairs 17-24, AND White 8-well strip tube with index pairs 25-32	Clear 96-well plate with index pairs 1–96 - - OR - - Blue 96-well plate with index pairs 97–192

Index Primer Pair Strip Tube and Plate Maps

- The black strip contains Index Primer Pairs 1-8, with pair #1 supplied in the well proximal to the numeral ***1*** etched on the strip’s plastic end tab.
- The blue strip contains Index Primer Pairs 9-16, with pair #9 supplied in the well proximal to the numeral ***9*** etched on the strip’s plastic end tab.
- The red strip contains Index Primer Pairs 17-24, with pair #17 supplied in the well proximal to the numeral ***17*** etched on the strip’s plastic end tab.
- The white strip contains Index Primer Pairs 25-32, with pair #25 supplied in the well proximal to the numeral ***25*** etched on the strip’s plastic end tab.

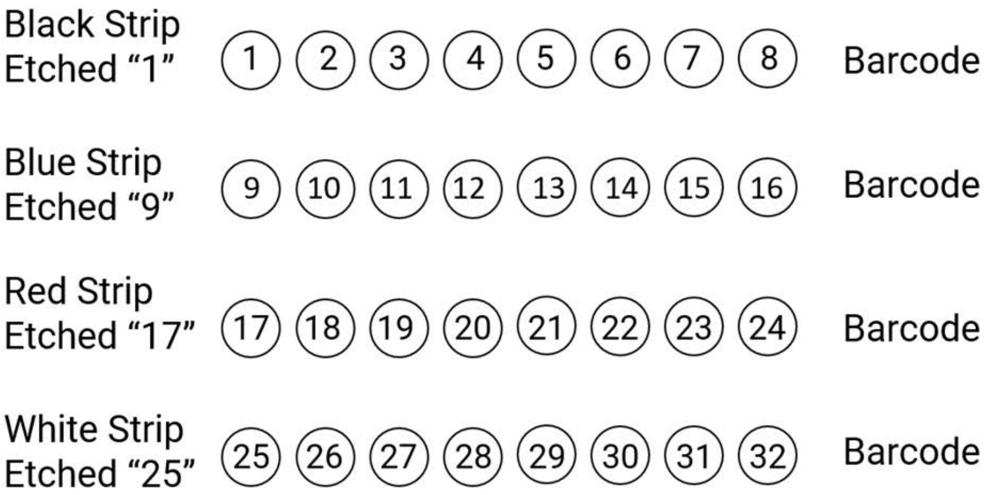

Figure 7 Map of the Avida Index Primer Pairs strip tubes provided with 16 reaction kits

Table 34 and Table 35 show the plate positions of the Avida Index Primer Pairs provided with 96 reaction kits.

Table 34 **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 35 **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 an Avida DNA Custom AddOn panel in the hybridization, make the protocol adjustments described below.

CAUTION Make sure that the Custom AddOn panel is compatible with the Avida catalog panel you are using in the workflow. If needed, check with Agilent Technical Support or your local sales representative for design ID compatibility. Custom AddOn panels are limited to 250 kb in size.

During preparation of the Hyb Mix 1 on page 25, substitute the reagents and volumes in Table 15 with those shown in Table 36 below.

If using the Avida DNA Focused Cancer Panel (design ID D3483231) as the Avida DNA catalog panel, make sure that the Custom AddOn panel is <20 kb. Consider designing a Custom Avida DNA Panel if an add-on larger than 20 kb is required.

Table 36 Preparation of Hyb Mix 1 using Avida DNA Custom AddOn Panel

Reagent	**Volume for 1 reaction**	**Volume for 8 reactions (includes excess)**	**Volume for 16 reactions (includes excess)**
Nuclease-Free Water	13.5 µL	121.5 µL	243 µL
Avida DNA Panel (catalog)	4 µL	36 µL	72 µL
Avida DNA Custom AddOn Panel	4 µL	36 µL	72 µL
Hyb Blocker	2.5 µL	22.5 µL	45 µL
Total	**24 µL**	**216 µL**	**432 µL**

Troubleshooting Guide

If yield of libraries is lower than previous captures

- Make sure that the Library Binding Beads (used in “Step 3. Library bead binding” on page 25) have equilibrated to room temperature before use.
- During “Step 1. Indexing PCR” on page 35, the number of cycles used in for Amplification stage 2 may require optimization. Refer to the *Note* on page 36 for guidelines.
- Take care throughout the workflow to minimize sample loss during pipetting and transfer steps.
- When mixing samples with beads, make sure the reactions are well mixed.

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, then wipe down with water to remove bleach residue. Review “Important practices for preventing contamination” on page 10.

If low percent on-target is observed in library sequencing results

- During “Step 3. Library bead binding” on page 25, include the optional second wash (see step 10 on page 26) and make sure to remove all residual Library Wash Buffer during the washes. The following techniques can be used to collect residual supernatant at the bottom of the tubes:

  For magnets that collect beads on tube sides: Tap the magnet stand on the bench 5 times.

  For magnets that collect beads at tube bottoms: Remove tubes from magnet and briefly spin. Return tubes to magnet before removing liquid.
- Following hybridization, make sure that the Capture Beads are fully resuspended during the wash steps with Hyb Wash Buffer 1 and Hyb Wash Buffer 2 and make sure to remove all residual wash buffer (see “Step 4. Washing” on page 33). Refer to techniques above for collecting residual supernatant based on magnet type.

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 19 on page 29. Additionally, make sure that the thermal cycler performs the washing incubation at 60°C as directed in Table 21 on page 32.
- When preparing Hyb Mix 2, make sure to use the correct formulation for your panel size. Table 17 on page 28 lists the formulation for panels <500 kb. Table 18 on page 28 lists the formulation for panels ≥500 kb.

Quick Reference Protocol

An abbreviated summary of the protocol steps is provided below for experienced users. Use the complete protocol until you are familiar with all of the protocol details such as reagent mixing instructions and instrument settings.

A new print-ready, graphics-based quick start protocol is available on the Agilent website: https://www.agilent.com/cs/library/usermanuals/public/G9409-90500.pdf.

Step	Summary of Conditions
Before starting a protocol: Pre-heat Hyb Wash Buffer 1	Heat stock bottle to 50°C in water bath or heat block In a 50°C water bath: Heat for 10 minutes, then transfer the bottle to a 50°C heat block until use On a 50°C heat block: Leave the bottle on the 50° heat block until use
	PCR-free Library Prep
Prepare End Prep Mix	**Per 8 reactions:** 63 µL End Prep Buffer + 27 µL End Prep Enzyme **Per 16 reactions:** 126 µL End Prep Buffer + 54 µL End Prep Enzyme Keep on ice
End-Repair and dA-Tail the DNA fragments	50 µL DNA (cfDNA or fragmented gDNA) + 10 µL End Prep Mix Mix and spin, then keep on ice Incubate in thermal cycler (heated lid 75°C): 30 min @ 20°C, 30 min @ 65°C, Hold @ 4°C
Prepare Ligation master mix	**Per 8 reactions:** 225 µL Ligation Buffer + 54 µL Ligation Enzyme **Per 16 reactions:** 450 µL Ligation Buffer + 108 µL Ligation Enzyme
Ligate adaptor	Add 5 µL Adaptor for ILM to each sample Then, add 31 µL Ligation Master Mix Mix and spin Incubate in thermal cycler (heated lid off): 30 min @ 20°C, Hold @ 4°C
Prepare reagents	**Library Binding Beads:** Room temperature for at least 15 minutes **Hyb Blocker and Hyb Enhancer:** Thaw then keep at room temperature **Avida DNA Panel:** Thaw then keep on ice **Hyb Buffer:** 37°C for at least 10–20 minutes, then keep at room temperature
Bind to Library Binding Beads	Vortex Library Binding Beads, let sit for 5 minutes Add 87 µL Library Binding Beads to each sample Mix and spin without letting beads settle Incubate 10 min @ room temperature
During 10-min incubation: Prepare Hyb Mix 1	**Per 8 reactions:** 157.5 µL Nuclease-free Water + 36 µL Avida DNA Panel + 22.5 µL Hyb Blocker **Per 16 reactions:** 315 µL Nuclease-free Water + 72 µL Avida DNA Panel + 45 µL Hyb Blocker Keep at room temperature
Wash	After 10-min incubation, place tubes on magnet, discard supernatant Wash beads in 180 µL Library Wash Buffer Place tubes on magnet, discard supernatant (Optional) Repeat wash with Library Wash Buffer Remove ALL remaining supernatant Remove samples from magnet
	Target Capture
Add Hyb Mix 1	Add 24 µL Hyb Mix 1 Mix and spin

Step	Summary of Conditions
Prepare Hyb Mix 2	Panels <500 kb **Per 8 reactions:** 270 µL Hyb Buffer + 54 µL Hyb Enhancer **Per 16 reactions:** 540 µL Hyb Buffer + 108 µL Hyb Enhancer Panels ≥500 kb **Per 8 reactions:** 279 µL Hyb Buffer + 45 µL Hyb Enhancer **Per 16 reactions:** 558 µL Hyb Buffer + 90 µL Hyb Enhancer
Hybridize	Add 36 µL Hyb Mix 2 Mix and spin without letting beads settle Incubate in thermal cycler (heated lid 103°C): 2 min @ 98°C, ≥2.5°C/second ramp down to 60°C, 60 min @ 60°C, Hold @ 4°C
During hybridization program: Prepare Capture Beads	Mix Capture Beads well Calculate volume of Capture Beads needed (8 µL/sample + overage) Transfer that volume into a 1.5-mL tube or into one or more PCR tubes Place tube on magnet, discard supernatant, then remove tube from magnet Wash beads 2× in 100 µL preheated Hyb Wash Buffer 1, discard supernatant after each wash (If processing >16 reactions, scale the volume of Hyb Wash Buffer 1 to use 100 µL per 16 reactions; see Table 20 on page 30) Remove tube from magnet Resuspend Capture Beads in Hyb Buffer at original volume calculated above, and mix Add 8 µL resuspended Capture Beads to each tube of a fresh strip tube
Bead capture	After hybridization, spin tubes and place on magnet Transfer each supernatant to tubes containing 8 µL-aliquots of prepared Capture Beads Mix and spin without letting beads settle Incubate in thermal cycler held @60°C (heated lid 75°C) After 10 minutes @ 60°C, remove tubes but leave thermal cycler running Place tubes on magnet, discard supernatant Remove samples from magnet
Wash	Wash beads in 150 µL preheated Hyb Wash Buffer 1 Place tubes on magnet and discard supernatant Remove samples from magnet Add 100 µL Hyb Wash Buffer 1 and mix Transfer each sample to a fresh strip tube and cap each tube Incubate in thermal cycler held @60°C (heated lid 75°C) After 3 minutes, place on magnet, discard supernatant Remove samples from magnet Wash beads in 150 µL Hyb Wash Buffer 2 Place tubes on magnet and discard all residual supernatant
Resuspend	Remove samples from magnet Add 20 µL Resuspension Buffer Mix gently and spin
	Indexing PCR, Library Purification, and Quality Assessment
Set up PCR reactions	Add 25 µL 2X Amplification Mastermix Add 5 µL primer pair Mix and spin
Perform indexing PCR	Incubate in thermal cycler (heated lid 103°C): 45 sec @98°C 5 cycles: 10 sec @98°C, 30 sec @ 63°C, 30 sec @72°C Additional cycles (as described in Table 25): 10 sec @98°C, 1 min @72°C 1 min @72°C Hold @4°C

Step	Summary of Conditions
Purify libraries	Add 50 µL of AMPure XP Beads and mix Incubate 5 min @ room temperature Place on magnet, discard supernatant, keep samples on magnet Wash beads 2× in 200 µL 80% ethanol, discard supernatant after each wash Remove all residual ethanol then air dry the beads up to 3 minutes (on magnet, lids open) Remove samples from magnet Add 23 µL of 1X Low TE Buffer Mix and spin Incubate at room temperature for 2 min then put samples back on magnet Transfer the eluate to fresh tube
Assess quality and quantity	(Optional) Quantification using fluorescence-based method (e.g., Qubit) Quality assessment on TapeStation or Fragment Analyzer Libraries are ready for sequencing or storage (4°C overnight or at –20°C for prolonged storage)

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In This Book

This guide provides instructions for using Avida DNA Reagent Kits for library preparation and target capture.

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