



# Avida Duo Methyl Reagent Kits

For targeted DNA sequencing and methylation sequencing on the Illumina platform

Library preparation, target captures, soft conversion, and indexing

## Protocol

**Version C1, November 2025**

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# 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 Duo Methyl 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 Duo workflow.

### 3 PCR-Free Library Preparation

This chapter describes the steps for the PCR-free library preparation section of the Avida Duo workflow (section 1 in Figure 2 on page 11). NGS libraries are prepared using adaptors that include Unique Molecular Identifiers (UMIs).

### 4 First Target Capture (Targeted Sequencing)

This chapter describes the steps for the first target capture section of the Avida Duo workflow (section 2A in Figure 2 on page 11). The adaptor-ligated libraries are hybridized with an Avida DNA panel. After hybridization, the targeted molecules are captured on Capture Beads coated with Streptavidin. Supernatants containing uncaptured DNA are set aside for the second target capture section of the workflow.

### 5 Indexing PCR for Targeted Sequencing

This chapter describes the steps for the indexing PCR section of the Avida Duo workflow (section 4A in Figure 2 on page 11) in which the captured libraries from the first target capture are amplified and indexed.

### 6 Second Target Capture (Methyl Sequencing)

This chapter describes the steps for the second target capture section of the Avida Duo workflow (section 2B in Figure 2 on page 11). The supernatant samples from the first capture (containing uncaptured DNA) are hybridized with an Avida Methyl panel. After hybridization, the targeted molecules are captured on Capture Beads coated with Streptavidin.

### 7 Soft Conversion and Repair

This chapter describes the steps for the soft conversion and repair section of the Avida Duo workflow (section 3 in Figure 2 on page 11). The adaptor-ligated libraries from the second target capture are treated with soft conversion reagents to deaminate any unmethylated cytosines, thereby converting them to uracils. The converted DNA is then purified, washed, and repaired. During the PCR indexing step (Chapter 8, "Indexing PCR for Methyl Sequencing") the uracils are replaced with thymines in the PCR products.

### 8 Indexing PCR for Methyl Sequencing

This chapter describes the steps for the indexing PCR section of the Avida Duo workflow (section 4B in Figure 2 on page 11) in which the converted libraries created from the second target capture are amplified and indexed.

## 9 Library Purification and Quality Assessment

This chapter describes the steps for the library purification and quality assessment section of the Avida Duo workflow (section 5 in Figure 2 on page 11). The captured libraries from both target captures are purified with AMPure XP Beads. Then, the purified, indexed libraries are quantified and analyzed for quality.

## 10 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.

## 11 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 C1

- Updated information on Avida panel sizes. See [Table 3](#) and [Table 4](#) on page 15 and the accompanying *Note* below the tables.
- Simplified preparation of the Library Binding Beads that eliminates 5-minute incubation of the stock tube/bottle prior to use. See [step 2](#) on [page 27](#).
- Increased excess volumes provided in [Table 35](#) on page 50 for preparation of 0.1X Soft Conversion Elution Buffer.
- Added instruction to reheat the Hyb Buffer prior to use if a precipitate forms during its storage at room temperature. See [step 2](#) on [page 30](#) and [step 1](#) on [page 43](#).
- Additional sequencing run guidelines, including Illumina Kit Configuration Selection Guidelines, added to "[Step 3. Sequence the libraries](#)" on page 67.
- Updated kit configuration information for Soft Conversion Reagent B, which may be shipped as a separate part number or included in the Avida Methyl and Duo Reagent Box 3. See [Table 2](#) on page 14 and [Table 51](#) on page 73.

## What's New in Version B1

- Support for new Avida DNA catalog panels (see [Table 3](#) on page 15 for part numbers).
- Additional information on Avida panel size values and calculations. See [Table 3](#) and [Table 4](#) on page 15, including the accompanying *Note*, for details.
- Correction to the custom AddOn panel size limit provided on [page 77](#).

## What's New in Version B0

- On cover page and throughout Protocol, using product title *Avida Duo Methyl reagent kit*.
- Throughout Protocol, using the term *soft conversion* to refer to the Avida soft conversion process for deamination of unmethylated cytosines.
- Support for new Avida DNA Discovery Cancer Panel (see [Table 3](#) on page 15 for part number).
- Expanded information on compatible reagent kits for the Agilent Fragment Analyzer system, including addition of electropherogram images in [Figure 7](#) through [Figure 10](#).
- In [Quick Reference Protocol](#), clarified storage conditions for the Avida panels and the final sequencing libraries, and corrected step names in the Second Target Capture section.
- In [Table 17](#) on page 29, corrected the storage temperature of Hyb Mix 1 and the kit name for Avida Duo Methyl Reagent Box 1.
- Added Note ([page 27](#)) on preparing aliquots of Hyb Mix 1.
- Expanded recommended Qubit Assay Kits to include Qubit dsDNA HS and BR Assay Kits.
- Updated thawing conditions for Adaptor for ILM ([page 22](#) and [page 24](#)).
- In the estimated time requirements provided in [Table 1](#) on page 11, the indexing PCR steps were corrected to 1 hour.
- Updated the instructions for "[Step 2. Capture Bead Preparation for First Target Capture](#)" on page 32 and "[Step 2. Capture Bead Preparation for Second Target Capture](#)" on page 43, including adding details based on number of reactions.
- Updated storage conditions for the Stopping Point on [page 58](#).
- In the [Quick Reference Protocol](#), added link to the new graphics-based Avida Duo Quick Start Protocol.
- In "[Step 3. Bead Capture for Second Target Capture](#)" on page 45, removed an unnecessary incubation on the magnet prior to adding the prepared Capture Beads.

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# 1

## Before You Begin

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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.

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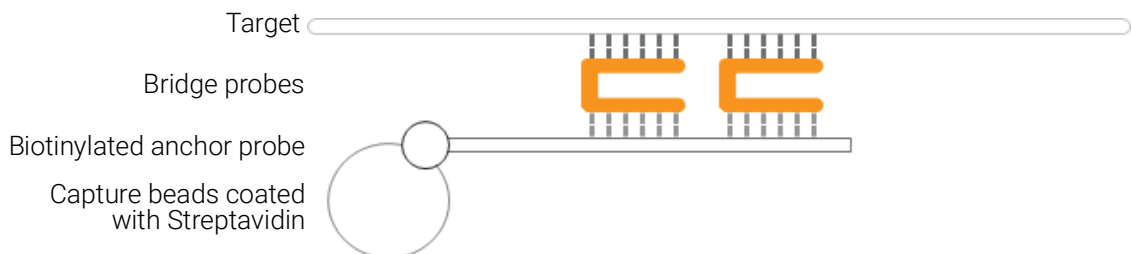
## Introduction

DNA genomic and epigenomic changes underlie the mechanisms of many physiological and pathological conditions, including cancer. The Avida Duo Methyl reagent kits are based on a novel technology developed and perfected to retrieve genomic and methylation changes from the same input without sample splitting. Compared to existing products for targeted sequencing and target methylation sequencing, the Avida Duo technology offers best-in-class recovery, which translates into superior sensitivity, especially at very low DNA input. The Avida Duo kits are particularly optimized to work with circulating cell-free DNA (cfDNA). The technology also supports fragmented genomic DNA (gDNA) isolated from fresh/fresh-frozen samples or from FFPE-derived samples. With a simple and fast workflow and unparalleled sensitivity, Avida Duo is poised to advance both research and clinical applications by enabling the next level accessibility to your liquid biopsy samples.

### 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 Duo 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 16 for detailed requirements on the DNA starting material).

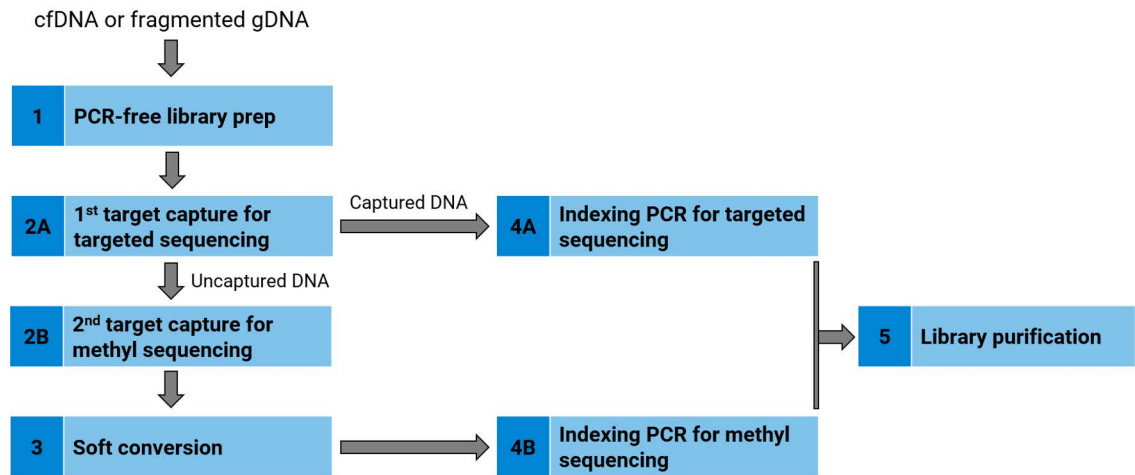
The workflow features a PCR-free library preparation with a proprietary pre-amplification target capture process that maximizes capture efficiency, specificity, and uniformity. It also features our unique soft conversion – a next-generation bisulfite conversion method – to give you the most sample recovery possible. Altogether, this streamlined protocol delivers high conversion and library complexity which enables highly sensitive detection of genomic changes and methylation changes.

The workflow has five sections (see [Figure 2](#) and [Table 1](#)). You can expect the entire process to take approximately 8 hours to complete (with some sections running concurrently). The resulting libraries are ready for next-generation sequencing (NGS) on Illumina platforms.

The Avida Duo workflow can be performed in a single day or spread across two days. The 1-day protocol can be completed in 8.5 hours by performing certain workflow sections concurrently. Notes are provided throughout the instructions to advise you on the recommended order of sections for completion of a single day protocol. For a 2-day protocol, complete the sections in the order presented in this Protocol, and refer to [Table 1](#) for options for overnight stopping points.

## Before You Begin

### Overview of the Workflow



**Figure 2** Diagram of the Avida Duo workflow sections

**Table 1** Avida Duo workflow sections and estimated time requirements for up to 8 samples

Section Number and Name	Description	Chapter	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	<a href="#">Chapter 3</a>	2 hours
2A 1st target capture for targeted sequencing	This target capture enriches specific targets by hybridization to the Avida DNA panel followed by washing of the captured targets	<a href="#">Chapter 4</a>	1.5 hours
4A Indexing PCR for targeted sequencing	Indexing PCR on 1st capture products to incorporate sample indexes and P5/P7 sequences necessary for Illumina sequencing <b>2-day protocol, option 1:</b> Store the indexed samples at 4°C overnight. See the <a href="#">Stopping Point</a> on <a href="#">page 40</a> .	<a href="#">Chapter 5</a>	1 hour
2B 2nd target capture for methyl sequencing	This target capture enriches another set of specific targets by hybridization to the Avida Methyl panel followed by washing of the captured targets <b>2-day protocol, option 2:</b> Run the hybridization thermal cycler program overnight. See <a href="#">Table 29</a> on <a href="#">page 42</a> .	<a href="#">Chapter 6</a>	1.5 hours
3 Soft conversion	Conversion of the targets captured in the 2nd target capture, followed by repair and purification	<a href="#">Chapter 7</a>	1.5 hours
4B Indexing PCR for methyl sequencing	Indexing PCR on converted libraries to incorporate sample indexes and P5/P7 sequences necessary for Illumina sequencing	<a href="#">Chapter 8</a>	1 hour
5 Library purification	Purification and quality assessment of sequencing libraries (both captures)	<a href="#">Chapter 9</a>	0.5 hours

## Procedural and Safety Notes

### Important practices for preventing contamination

The Avida Duo 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.

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**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.
- 

**CAUTION**

To reduce the risk of contamination from an unconverted library to a converted library, change gloves at the specified steps in the protocol. Agilent strongly recommends separating the places where related equipment and supplies for conversion are located from the laboratory space where pre-PCR library prep and target capture is performed.

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### Safety notes

**WARNING**

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

In general, follow Biosafety Level 1 (BSL1) safety rules.

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## 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](#) and [Chapter 6](#)):
  - 1 Pre-aliquot reagents to a pipetting reservoir of appropriate size or to the tubes of a fresh strip tube.
  - 2 Use a multichannel pipette for washes.
- **Because the Avida Duo 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  $-20^{\circ}\text{C}$  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  $-20^{\circ}\text{C}$  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  $4^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$ , are marked in the protocol.

## Materials Provided with Avida Duo Methyl Reagent Kits and Panels

**Table 2.** lists the Avida Duo Methyl 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 11**, “Reagent Kit Contents,” starting on page 72.

**Table 3** lists the Avida DNA panels compatible with the 1st target capture for targeted sequencing. **Table 4** lists the Avida Methyl panel compatible with the 2nd target capture for methyl sequencing.

**Table 2** Avida Duo Methyl reagent kits

Avida Duo Methyl reagent kits	Sub-kit boxes	Storage Temperature
P/N G9439A Avida Duo Methyl reagent kit with 1-32 index primer pairs for ILM, 16 reactions	Avida Duo Methyl Reagent Box 1, 16 Reactions	-20°C
	Avida DNA and Duo Reagent Box 2, 16 Reactions	Room temperature
	Avida Methyl and Duo Reagent Box 3, 16 Reactions	Room temperature
	Avida Duo Beads Box, 16 Reactions	4°C
	Avida Index Primer Pairs 1–32 for ILM	-20°C
	Soft Conversion Reagent B*	Room temperature
P/N G9440A Avida Duo Methyl reagent kit with 1-192 index primer pairs for ILM, 96 reactions	Avida Duo Methyl Reagent Box 1, 96 Reactions	-20°C
	Avida DNA and Duo Reagent Box 2, 96 Reactions	Room temperature
	Avida Methyl and Duo Reagent Box 3, 96 Reactions	Room temperature
	Avida Duo Beads Box, 96 Reactions	4°C
	Avida Index Primer Pairs 1–192 for ILM	-20°C
	Soft Conversion Reagent B*	Room temperature

\* Soft Conversion Reagent B may be shipped as part of the Avida Methyl and Duo Reagent Box 3 or it may be shipped in its own separate bag at room temperature.

**Table 3** Compatible Avida DNA panels for the 1st target capture

Capture Probes Panel	Target Size	Probes Size	Design ID	P/N for 16-reactions	P/N for 96-reactions
<b>Pre-designed Avida DNA Panels</b>					
Avida DNA Discovery Cancer Panel	1.96 Mb	3.43 Mb	D3496941	5280-0040	5280-0044
Avida DNA Expanded Cancer Panel	344 kb	507 kb	D3483241	5280-0046	5280-0047
Avida DNA Focused Cancer Panel	26 kb	41 kb	D3483231	5280-0049	5280-0050
Avida DNA Onco LB Panel	875 kb	1.17 Mb	D3505061	5280-0171	5280-0172
Avida DNA Onco LB Plus Panel	1.75 Mb	2.48 Mb	D3505071	5280-0174	5280-0175
Avida DNA Lymphoma Panel	379 kb	451 kb	D3505051	5280-0177	5280-0178

**Table 4** Compatible Avida Methyl panels for the 2nd target capture

Capture Probes Panel	Target Size	Probes Size	Design ID	P/N for 16-reactions	P/N for 96-reactions
Avida Methyl 3400 DMR Cancer Panel	647 kb	743 kb	M3483261	5280-0058	5280-0059

**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.

In the Avida Methyl 3400 DMR Cancer Panel, the differentially methylated regions used as the targets have a significant degree of overlap. Refer to the *Regions.bed* file for unmerged individual target annotation.

Certain protocol steps vary based on the panel (i.e., preparing the Hyb Mix 2 on [page 30](#) and programming the thermal cycler for indexing PCR on [page 39](#) and [page 55](#)). 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 Duo Methyl 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 5](#). 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 2](#), “gDNA Fragmentation” for instructions). Since cfDNA typically has an average size of 170 base pairs, no further fragmentation is required.

**Table 5 Starting material requirements by DNA sample type**

Sample type	Input quantity	Fragmentation requirements	Storage buffer
cfDNA	3–100 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 <sup>†</sup>	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 3 ng. For detection of methylation changes, increasing the input improves performance.

<sup>†</sup> 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 6](#) through [Table 8](#) for additional reagents and equipment required to complete the workflow.

**Table 6 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 7** 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 <a href="#">Table 8</a> on page 17
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 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:	
<i>Select from the following kits*</i>	
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 high-quality gDNA samples and FFPE-derived 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 gDNA Fragmentation

Step 1. Prepare for shearing [20](#)

Step 2. Shear the gDNA samples [20](#)

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

### NOTE

Fragmentation is only required for gDNA samples. If you are using cfDNA samples, proceed to [Chapter 3](#), "PCR-Free Library Preparation."

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## Step 1. Prepare for shearing

### NOTE

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

- 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  $\mu$ L. 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- $\mu$ L 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 10](#).

**Table 10** 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 $\times$ 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  $\mu$ L) to a strip tube sample well. Keep the samples on ice.

**gDNA Fragmentation****Step 2. Shear the gDNA samples**

- 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- $\mu$ L 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

Step 1. End prep (end repair and dA-tailing) [23](#)

Step 2. Ligation [25](#)

Step 3. Library bead binding [27](#)

This chapter describes the steps for the PCR-free library preparation section of the Avida Duo workflow (section 1 in [Figure 2](#) on page 11). NGS libraries are prepared using adaptors that include Unique Molecular Identifiers (UMIs).

See [Table 5](#) on page 16 for DNA sample input requirements.

This chapter uses the reagents listed in [Table 11](#).

**Table 11** 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 Duo Methyl Reagent Box 1, stored at $-20^{\circ}\text{C}$
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 Duo Beads Box, stored at $4^{\circ}\text{C}$
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 $-20^{\circ}\text{C}$

**CAUTION**

Review the [“Procedural and Safety Notes”](#) on page 12 before getting started.

Because the Avida Duo 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.

**PCR-Free Library Preparation****Step 1. End prep (end repair and dA-tailing)****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](#) and [Chapter 6](#). 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.

If you are performing [Chapter 4](#) and [Chapter 6](#) on the same day, return the bottle to the 50°C heat block after its use in [Chapter 4](#) until it is needed again in [Chapter 6](#).

**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 5](#) on page 16 for complete information on the DNA sample input requirements. Keep DNA samples on ice.
  - cfDNA samples: 3–100 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 12](#) 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 or use it immediately. The End Prep Mix is stable for up to 2 hours at 4°C.

**Table 12** 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
<b>Total</b>	<b>10 µL</b>	<b>90 µL</b>	<b>180 µL</b>

**PCR-Free Library Preparation****Step 1. End prep (end repair and dA-tailing)**

- 4 Add 10  $\mu$ L 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  $\mu$ L. 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 13](#). Set the heated lid to 75°C. Load the strip tubes and run the program.

**Table 13** 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 14](#) 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 or use it immediately.

### 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 14** 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
<b>Total</b>	<b>31 µL</b>	<b>279 µL</b>	<b>558 µL</b>

- 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 15](#) with the heated lid off. Load the strip tubes and run the program.

**Table 15** Thermal cycler program for ligation

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

## PCR-Free Library Preparation

### Step 2. Ligation

- 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](#), “First Target Capture (Targeted Sequencing)” and [Chapter 6](#), “Second Target Capture (Methyl Sequencing).”

#### 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 DNA 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 DNA Panel are used to prepare Hyb Mix 1 in [Table 16](#). The Hyb Enhancer is used in [Chapter 4](#), “First Target Capture (Targeted Sequencing)” and [Chapter 6](#), “Second Target Capture (Methyl Sequencing).”

#### CAUTION

The Hyb Enhancer must be thawed at room temperature. Once thawed, thoroughly vortex the Hyb Enhancer to solubilize any precipitates.

## 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.  
  
Note that some bead aggregates may remain even after vortexing. The bead aggregates do not impact performance.
- 3 Add 87  $\mu\text{L}$  (~0.9X volume) of Library Binding Beads to each adaptor-ligated DNA sample, bringing the sample volume to 183  $\mu\text{L}$ . Mix by pipetting up and down 15–20 times using a pipette set to 150  $\mu\text{L}$ . 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 16](#) 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  $\mu\text{L}$  of Hyb Mix 1 into the tubes of a fresh strip tube (one tube per sample). Then, during [step 1](#) on [page 30](#), use a multichannel pipette to transfer 24  $\mu\text{L}$  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 16** 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 $\mu\text{L}$	157.5 $\mu\text{L}$	315 $\mu\text{L}$
Avida DNA Panel	4 $\mu\text{L}$	36 $\mu\text{L}$	72 $\mu\text{L}$
Hyb Blocker	2.5 $\mu\text{L}$	22.5 $\mu\text{L}$	45 $\mu\text{L}$
<b>Total</b>	<b>24 <math>\mu\text{L}</math></b>	<b>216 <math>\mu\text{L}</math></b>	<b>432 <math>\mu\text{L}</math></b>

- 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.**

**PCR-Free Library Preparation****Step 3. Library bead binding**

- 8 Add 180  $\mu$ L 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 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  $\mu$ L pipette.

Refer to [“Procedural notes”](#) on page 13 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 30](#) (in [Chapter 4](#), “First Target Capture (Targeted Sequencing)”) and add the Hyb Mix 1 that you prepared earlier to make sure that the beads do not dry out.

## 4 First Target Capture (Targeted Sequencing)

- Step 1. Hybridization for First Target Capture [30](#)  
 Step 2. Capture Bead Preparation for First Target Capture [32](#)  
 Step 3. Bead Capture for First Target Capture [34](#)  
 Step 4. Washing for First Target Capture [35](#)

This chapter describes the steps for the first target capture section of the Avida Duo workflow (section 2A in [Figure 2](#) on page 11). The adaptor-ligated libraries are hybridized with an Avida DNA panel. After hybridization, the targeted molecules are captured on Capture Beads coated with Streptavidin. Supernatants containing uncaptured DNA are set aside for the second target capture section of the workflow.

This chapter uses the reagents listed in [Table 17](#).

**Table 17** 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 Duo Methyl 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 Duo 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 ( <a href="#">page 27</a> ) and kept at room temperature until use.	

## Step 1. Hybridization for First Target Capture

- 1 Add 24  $\mu\text{L}$  of Hyb Mix 1 (prepared on [page 27](#)) 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 27](#)) you distributed 26  $\mu\text{L}$  of Hyb Mix 1 into the tubes of a fresh strip tube, you can now use a multichannel pipette to transfer 24  $\mu\text{L}$  from the strip tube to the beads bound with adaptor-ligated library.

- 2 Thoroughly vortex the Hyb Buffer that has been kept at room temperature.  
If a precipitate is visible in the Hyb Buffer, reheat to 37°C (as described on [page 26](#)) to resolubilize prior to use.
- 3 Prepare the Hyb Mix 2 using the volumes in either [Table 18](#) or [Table 19](#). Select the appropriate table based on the Avida DNA panel to be used in the protocol.

Use [Table 18](#) for the following Avida DNA panels.

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

Use [Table 19](#) for the following Avida DNA panels.

- Avida DNA Discovery Panel, Onco LB Panel, and Onco LB Plus 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 in this chapter.

If performing the second target capture within the same day, keep the Hyb Enhancer at room temperature until needed for that step.

**Table 18** Preparation of Hyb Mix 2 for Expanded, Focused, and Lymphoma

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
Hyb Buffer	30 $\mu\text{L}$	270 $\mu\text{L}$	540 $\mu\text{L}$
Hyb Enhancer	6 $\mu\text{L}$	54 $\mu\text{L}$	108 $\mu\text{L}$
<b>Total</b>	<b>36 <math>\mu\text{L}</math></b>	<b>324 <math>\mu\text{L}</math></b>	<b>648 <math>\mu\text{L}</math></b>

**Table 19** Preparation of Hyb Mix 2 for Discovery, Onco LB, and Onco LB Plus

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
Hyb Buffer	31 $\mu\text{L}$	279 $\mu\text{L}$	558 $\mu\text{L}$
Hyb Enhancer	5 $\mu\text{L}$	45 $\mu\text{L}$	90 $\mu\text{L}$
<b>Total</b>	<b>36 <math>\mu\text{L}</math></b>	<b>324 <math>\mu\text{L}</math></b>	<b>648 <math>\mu\text{L}</math></b>

## First Target Capture (Targeted Sequencing)

### Step 1. Hybridization for First Target Capture

- 4 Add 36  $\mu\text{L}$  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  $\mu\text{L}$ .
- 5 Mix by pipetting up and down 15–20 times using a pipette set to 50  $\mu\text{L}$ . 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 20](#). Set the heated lid to 103°C. Load the strip tubes and run the program.

**Table 20** Thermal cycler program for hybridization

Step	Temperature	Time
Step 1	98°C	2 minutes
Step 2	$\geq 2.5^\circ\text{C}/\text{second}$ ramp down to 60°C	
Step 3	60°C	60 minutes
Step 4	60°C	Hold

#### NOTE

The thermal cycler program in [Table 20](#) uses a 60-minute hybridization which is sufficient for most applications. For panels >500 kb, a longer hybridization time (up to 16 hours) may improve capture efficiency.

## Step 2. Capture Bead Preparation for First Target Capture

### 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 for First Target Capture.”](#)
- 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 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 21](#). 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 21** 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.

**First Target Capture (Targeted Sequencing)****Step 2. Capture Bead Preparation for First Target Capture**

- 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).

If performing the second target capture within the same day, keep the Hyb Buffer at room temperature until needed for that step.

**NOTE**

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

- 9 Add 8  $\mu\text{L}$  of resuspended Capture Beads to each tube of a fresh strip tube (one for each hybridization reaction).

## Step 3. Bead Capture for First Target 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 22](#). Set the heated lid to 75°C.

**Table 22** 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- $\mu$ L aliquot of prepared Capture Beads (from [step 9](#) on [page 33](#)). 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.
- 6 **Transfer all 68  $\mu$ L of each supernatant to a fresh strip tube.** Set aside the supernatant samples until needed in [Chapter 6](#), “Second Target Capture (Methyl Sequencing).” Store at 4°C (for up to 2 hours) or –20°C (overnight).  
  
The supernatant samples contain DNA inserts that were not captured during the 1st target capture with the Avida DNA Panel. These samples will be used in the 2nd target capture with the Avida Methyl Panel.
- 7 Remove the strip tubes containing the Capture Beads/library DNA hybrids from the magnet. With these samples, immediately proceed to [“Step 4. Washing for First Target Capture.”](#)

## Step 4. Washing for First Target Capture

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 13 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  $\mu\text{L}$  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  $\mu\text{L}$  pipette to remove all residual Hyb Wash Buffer 1.
- 3 Remove the strip tubes from the magnet. Add 100  $\mu\text{L}$  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. *(Complete resuspension is critical.)* **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  $\mu\text{L}$  pipette to remove all residual Hyb Wash Buffer 1.  
Promptly execute the next steps ([step 7](#) and [step 8](#)) to avoid over-drying the bead pellets.
- 7 Remove the strip tubes from the magnet.
- 8 Add 150  $\mu\text{L}$  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. *(Complete resuspension is critical.)* 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  $\mu\text{L}$  pipette to remove all residual Hyb Wash Buffer 2.
- 10 Remove the strip tubes from the magnet.
- 11 Add 20  $\mu\text{L}$  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 for Targeted Sequencing.”

## First Target Capture (Targeted Sequencing)

### Step 4. Washing for First Target Capture

**CAUTION**

Do not discard the beads. The entire 20  $\mu$ L of resuspended beads with captured DNA will go into indexing PCR.

---

**NOTE**

**Tip for a 1-day protocol:** Store the samples in Resuspension Buffer at 4°C and proceed to [Chapter 6](#), “Second Target Capture (Methyl Sequencing).” Then, proceed to [Chapter 7](#), “Soft Conversion and Repair.”

When finished with the instructions in [Chapter 7](#), perform the indexing PCR for the targeted sequencing samples ([Chapter 5](#)) and the indexing PCR for the methyl sequencing samples ([Chapter 8](#)) on separate thermal cyclers that are running concurrently.

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## 5 Indexing PCR for Targeted Sequencing

Step 1. PCR Set Up (Targeted Sequencing) [38](#)

Step 2. Indexing PCR Program (Targeted Sequencing) [39](#)

This chapter describes the steps for the indexing PCR section of the Avida Duo workflow (section 4A in [Figure 2](#) on page 11) in which the captured libraries from the first target capture are amplified and indexed.

This chapter uses the reagents listed in [Table 23](#).

**Table 23** Reagents for indexing PCR for targeted sequencing

Reagent	Usage Notes	Kit/Storage Location
2X Amplification Mastermix (tube with white cap or bottle)	Thaw on ice.	Avida Duo Methyl Reagent Box 1, stored at $-20^{\circ}\text{C}$
Avida Index Primer Pairs	Primer pairs are provided in either a strip or a plate. Thaw strip or plate on ice.	Stored at $-20^{\circ}\text{C}$

## Step 1. PCR Set Up (Targeted Sequencing)

### CAUTION

Take precautions to avoid amplicon contamination during setup of the indexing PCR reactions. Review [“Important practices for preventing contamination”](#) on page 12.

- 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 35](#)), add 25  $\mu\text{L}$  of 2X Amplification Mastermix to each tube. Mix by pipetting up and down 15–20 times. Briefly spin.
- 3 Add 5  $\mu\text{L}$  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 24](#) summarizes the contents in each PCR reaction.

**Table 24** Indexing PCR reaction components

Reagent	Volume per PCR reaction
Resuspended beads with captured DNA (from <a href="#">step 11</a> on <a href="#">page 35</a> )	20 $\mu\text{L}$
2X Amplification Mastermix	25 $\mu\text{L}$
Avida Index Primer Pair	5 $\mu\text{L}$
<b>Total</b>	<b>50 <math>\mu\text{L}</math></b>

### 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 2. Indexing PCR Program (Targeted Sequencing)

- 1 Program the thermal cycler to run the indexing PCR thermal cycler program shown in [Table 25](#). Set the heated lid to 103°C. Load the strip tubes and run the program.

**Table 25** 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 <a href="#">Table 26</a> for recommendations	98°C	10 seconds
		72°C	1 minute
Final extension	1	72°C	1 minute
Final hold	1	4°C	Hold

**Table 26** 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 <ul style="list-style-type: none"> <li>• Avida DNA Discovery Cancer Panel: 11 cycles</li> <li>• Avida DNA Onco LB Plus Panel: 13 cycles</li> <li>• Avida DNA Expanded Cancer Panel: 14 cycles</li> <li>• Avida DNA Onco LB Panel: 14 cycles</li> <li>• Avida DNA Lymphoma Panel: 16 cycles</li> </ul>
>50 kb to 100 kb	17
10 kb to 50 kb	18 <ul style="list-style-type: none"> <li>• Includes the Avida DNA Focused Panel</li> </ul>
<10 kb	19

## Indexing PCR for Targeted Sequencing

### Step 2. Indexing PCR Program (Targeted Sequencing)

**NOTE**

The cycle number recommendations in [Table 26](#) 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 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**

- 3 Set aside the indexed libraries for targeted sequencing until needed in [Chapter 9](#), “Library Purification and Quality Assessment.” Store at 4°C (overnight) or –20°C (up to 72 hours).
- 4 Proceed to [Chapter 6](#), “Second Target Capture (Methyl Sequencing)” to perform the second target capture for methyl sequencing using the supernatant samples that were set aside in [step 6](#) on [page 34](#).

## 6 Second Target Capture (Methyl Sequencing)

- Step 1. Hybridization for Second Target Capture [42](#)
- Step 2. Capture Bead Preparation for Second Target Capture [43](#)
- Step 3. Bead Capture for Second Target Capture [45](#)
- Step 4. Washing for Second Target Capture [46](#)

This chapter describes the steps for the second target capture section of the Avida Duo workflow (section 2B in [Figure 2](#) on page 11). The supernatant samples from the first capture (containing uncaptured DNA) are hybridized with an Avida Methyl panel. After hybridization, the targeted molecules are captured on Capture Beads coated with Streptavidin.

This chapter uses the reagents listed in [Table 27](#).

**Table 27** 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 Duo Methyl 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 Duo Beads Box, stored at 4°C
Hyb Wash Buffer 1 (bottle)	Heat the stock bottle at 50°C in water bath or heat block until use.	Avida DNA and Duo Reagent Box 2, stored at Room Temperature
Hyb Wash Buffer 2 (bottle)	–	
Resuspension Buffer (tube with red cap or bottle)	–	
Avida Methyl 3400 DMR Cancer Panel	Thaw at room temperature then keep on ice.	Stored at –20°C

## Step 1. Hybridization for Second Target Capture

### NOTE

If Hyb Wash Buffer 1 is not already preheated to 50°C, begin that heating process before starting hybridization for the second target capture.

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

- 1 Prepare the Methyl Hyb Mix by combining the reagents in [Table 28](#) in a 1.5-mL tube. Gently vortex the Methyl Hyb Mix, then briefly spin the tube.

**Table 28** Preparation of Methyl Hyb Mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
Avida Methyl Panel	4.8 µL	43.2 µL	86.4 µL
Hyb Enhancer	1.5 µL	13.5 µL	27 µL
<b>Total</b>	<b>6.3 µL</b>	<b>56.7 µL</b>	<b>113.4 µL</b>

- 2 Add 6.3 µL of Methyl Hyb Mix to the supernatant samples that were set aside during the first target capture (see [step 6](#) on [page 34](#)). Mix by pipetting up and down 15–20 times. Briefly spin the strip tubes.
- 3 Program the thermal cycler to run the hybridization thermal cycler program shown in [Table 29](#). Set the heated lid to 103°C. Load the strip tubes and run the program.

**Table 29** 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 29](#) uses a 60-minute hybridization which is sufficient for most applications. A longer hybridization time (up to 16 hours) may improve capture efficiency, especially for panels >500 kb.

## Step 2. Capture Bead Preparation for Second Target Capture

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 2. Capture Bead Preparation for First Target Capture.”](#)

If a precipitate is visible in the Hyb Buffer, reheat to 37°C (as described on [page 26](#)) to resolubilize prior to use.

- 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 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 30](#). 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 30** 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).

**Second Target Capture (Methyl Sequencing)**

## Step 2. Capture Bead Preparation for Second Target Capture

**NOTE**

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

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## Step 3. Bead Capture for Second Target 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 31](#). Set the heated lid to 75°C.

**Table 31** Thermal cycler program for washing

Step	Temperature	Time
Step 1	60°C	Hold

- 2 Briefly spin the strip tubes. Then, add 8 µL of prepared Capture Beads to each tube (make sure the Capture Beads are well mixed prior to pipetting). Mix by pipetting up and down 15–20 times (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.
- 3 Perform bead binding by placing the strip tubes on the thermal cycler running at 60°C for 10 minutes.
- 4 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). Remove and discard all supernatant using a pipette set to 150 µL.  
  
Leave the thermal cycler running at 60°C.
- 5 Remove the strip tubes from the magnet and immediately proceed to [“Step 4. Washing for Second Target Capture.”](#)

## Step 4. Washing for Second Target Capture

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

Refer to “[Procedural notes](#)” on page 13 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  $\mu\text{L}$  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  $\mu\text{L}$  pipette to remove all residual Hyb Wash Buffer 1.
- 3 Remove the strip tubes from the magnet. Add 100  $\mu\text{L}$  of Hyb Wash Buffer 1 to each tube. Mix by pipetting up and down 15–20 times, making sure the beads are fully resuspended. *(Complete resuspension is critical.)* **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  $\mu\text{L}$  pipette to remove all residual Hyb Wash Buffer 1.  
  
Promptly execute the next steps ([step 7](#) and [step 8](#)) to avoid over-drying the bead pellets.
- 7 Remove the strip tubes from the magnet.
- 8 Add 150  $\mu\text{L}$  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. *(Complete resuspension is critical.)* Briefly spin. Proceed to [Chapter 7](#), “Soft Conversion and Repair.”

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

## 7 Soft Conversion and Repair

- Step 1. Soft Conversion [48](#)
- Step 2. Bead Purification [50](#)
- Step 3. Washing (Bead-Bound Converted Libraries) [51](#)
- Step 4. DNA Repair [52](#)

This chapter describes the steps for the soft conversion and repair section of the Avida Duo workflow (section 3 in [Figure 2](#) on page 11). The adaptor-ligated libraries from the second target capture are treated with soft conversion reagents to deaminate any unmethylated cytosines, thereby converting them to uracils. The converted DNA is then purified, washed, and repaired. During the PCR indexing step ([Chapter 8](#), “Indexing PCR for Methyl Sequencing”) the uracils are replaced with thymines in the PCR products.

This chapter uses the reagents listed in [Table 32](#).

**Table 32** Reagents for soft conversion and repair

Reagent	Usage Notes	Kit/Storage Location
Soft Conversion Reagent A (amber tube with amber cap)	Thaw at room temperature; vortex to mix.	Avida Duo Methyl Reagent box 1; stored at $-20^{\circ}\text{C}$
Soft Conversion Reagent B (amber tube with blue cap)	Vortex to mix; discard tube after use (do not re-use).	Stored at room temperature; either included with Avida Methyl and Duo Reagent Box 3 or shipped separately
Soft Conversion Binding Beads (bottle)	Vortex to mix.	Avida Methyl and Duo Reagent Box 3, stored at room temperature
5X Soft Conversion Wash Buffer (bottle)	Add ethanol prior to initial use.	
Soft Conversion Elution Buffer (tube with green cap)	—	
Soft Conversion Repair Solution (tube with yellow cap)	—	
Nuclease-Free Water (tube with clear cap or bottle)	—	
100% ethanol	—	Not provided

## Step 1. Soft Conversion

**CAUTION**

Agilent recommends designating a separate area for conversion work to avoid contamination with unconverted materials. Always change to a new pair of gloves for conversion work.

---

- 1 Briefly spin the strip tubes containing the Capture Beads/library DNA hybrids in Hyb Wash Buffer 2 (from the last step of [Chapter 6](#), “Second Target Capture (Methyl Sequencing)”).
- 2 Place the strip tubes on the magnet for 1 minute or until the solution becomes clear. Remove and discard the supernatant from each tube.
- 3 Collect all residual supernatant at the bottom of the tubes, then remove and discard the residual supernatant from each tube using a 20  $\mu\text{L}$  pipette. Immediately remove the tubes from the magnet.

**CAUTION**

Removing all residual Hyb Wash Buffer 2 from the beads is critical. Residual buffer may lower the conversion rate.

Do not leave the tubes on the magnet once the buffer is completely removed. Leaving dry beads on the magnet could cause the beads to aggregate, resulting in low recovery yield.

---

- 4 Add 11.5  $\mu\text{L}$  of Soft Conversion Elution Buffer to each tube. Gently pulse vortex to ensure the beads are fully resuspended in the solution. Spin the strip tubes for 1–2 seconds to collect the liquid at the bottom of the tube, making sure the beads do not settle.

**NOTE**

For gentle pulse vortexing, reduce the vortex speed to 50% power, then pulse vortex the tubes for 1–2 seconds at a time for a total of 10 times.

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**NOTE**

When handling multiple samples, Agilent recommends aliquoting 12.5  $\mu\text{L}$  of Soft Conversion Elution Buffer into the tubes of a fresh strip tube (one tube per sample). Then, use a multichannel pipette to transfer 11.5  $\mu\text{L}$  of the buffer from the strip tube to the beads bound with adaptor-ligated library. This technique helps prevent the beads from drying out.

---

- 5 Incubate the tubes at room temperature for 10 minutes.
- 6 During the 10-minute incubation, prepare the Soft Conversion Mix by combining the components in [Table 33](#) in a 1.5-mL tube.

Thoroughly vortex Soft Conversion Reagent A and Soft Conversion Reagent B before adding them to the mix. It is normal to see some white crystals forming around the tube and lid of Soft Conversion Reagent B.

**NOTE**

Each tube of Soft Conversion Reagent B is provided as a single-use reagent. Once the tube is opened, the solution is susceptible to oxidization. Discard the tube after use.

---

## Soft Conversion and Repair

### Step 1. Soft Conversion

Vortex the Soft Conversion Mix thoroughly, then briefly spin the tube. The Soft Conversion Mix needs to be prepared fresh each time and used within 10 minutes after preparation. Keep the cap of the tube closed as much as possible.

**Table 33** Preparation of Soft Conversion Mix

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
Soft Conversion Reagent A	3.5 $\mu$ L	31.5 $\mu$ L	63 $\mu$ L
Soft Conversion Reagent B	20 $\mu$ L	180 $\mu$ L	360 $\mu$ L
<b>Total</b>	<b>23.5 <math>\mu</math>L</b>	<b>211.5 <math>\mu</math>L</b>	<b>423 <math>\mu</math>L</b>

- 7 After the 10-minute incubation ([step 5](#), above), place the strip tubes on the magnet for 1 minute or until the solution becomes clear (up to 2 minutes).
- 8 **Carefully transfer the supernatant from each tube of the strip tube** (making sure to get all the supernatant) to a fresh strip tube.
- 9 Add 23.5  $\mu$ L of freshly prepared Soft Conversion Mix to each tube. Mix by pipetting up and down 15–20 times then immediately close the cap.
- 10 Program the thermal cycler to run the conversion thermal cycler program shown in [Table 34](#). Set the heated lid to 85°C. Load the strip tubes and run the program.

**Table 34** Thermal cycler program for conversion

Step	Temperature	Time
Step 1	75°C	25 minutes*
Step 2	10°C	Hold

\* If conversion >99.5% is desired for your assay, increase the duration of Step 1 from 25 minutes to 35 minutes. While this added duration maximizes conversion, it will reduce molecular recovery by 10–15%.

#### CAUTION

As soon as the thermal cycler program reaches the 10°C hold step, proceed immediately to [“Step 2. Bead Purification.”](#) Prolonged holding at 10°C may lower the recovery yield.

## Step 2. Bead Purification

- 1 Once the thermal cycler program for conversion reaches the 10°C hold step, remove the strip tubes from the thermal cycler.

### NOTE

You may see a brown coating in the tubes following conversion. This appearance is normal.

- 2 Thoroughly vortex the stock of Soft Conversion Binding Beads, then add 150 µL of beads to each of the tubes containing a converted library. Mix by pipetting up and down 15–20 times, making sure the beads are fully resuspended. Spin for 1 to 2 seconds to collect the liquid at the bottom of the tubes, making sure the beads do not settle.
- 3 Incubate the tubes at room temperature for 10 minutes.
- 4 During the 10-minute incubation, prepare a 0.1X dilution of the Soft Conversion Elution Buffer by combining the components in [Table 35](#) in a 1.5-mL tube.

The 0.1X Soft Conversion Elution Buffer is used in [“Step 4. DNA Repair”](#) on page 52.

**Table 35** Preparation of 0.1X Soft Conversion Elution Buffer

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
Soft Conversion Elution Buffer	1.5 µL	16.5 µL	33 µL
Nuclease-Free Water	13.5 µL	148.5 µL	297 µL
<b>Total</b>	<b>15 µL</b>	<b>165 µL</b>	<b>330 µL</b>

- 5 After the 10-minute incubation ([step 3](#), above), carefully place the strip tubes on the magnet for 30 seconds or until the solution becomes clear (up to 1 minute). Use a 20 µL pipette to remove and discard all supernatant.
- 6 Remove the tubes from the magnet and proceed immediately to [“Step 3. Washing \(Bead-Bound Converted Libraries\).”](#)

## Step 3. Washing (Bead-Bound Converted Libraries)

**NOTE**

Before using the Soft Conversion Wash Buffer for the first time, prepare a 1X stock by adding 100% ethanol to the stock bottle containing 5X Soft Conversion Wash Buffer. Vortex to mix, then mark the check box on the bottle to indicate that ethanol was added. Keep bottle tightly capped to avoid ethanol evaporation.

- The bottle of 5X Soft Conversion Wash Buffer supplied with the 16-reaction kits (p/n 5271-0115) requires 8 mL of 100% ethanol.
- The bottle of 5X Soft Conversion Wash Buffer supplied with the 96-reaction kits (p/n 5271-0142) requires 32 mL of 100% ethanol.

**CAUTION**

For all washing steps in this section, do NOT spin the tubes in a centrifuge. Handle the tubes carefully to avoid any splashing.

- 1 Add 150  $\mu$ L of 1X Soft Conversion Wash Buffer to each tube of beads. Mix by pipetting up and down 15–20 times.

When handling multiple samples, Agilent recommends pouring 1X Soft Conversion Wash Buffer into a reagent reservoir and pipetting it using a multichannel pipette.

- 2 **Transfer the contents of each tube to a fresh strip tube**, then place the fresh strip tube on the magnet for 30 seconds or until the solution becomes clear (up to 1 minute). Remove and discard the supernatant from each tube, then remove the tubes from the magnet.
- 3 Add 150  $\mu$ L of 1X Soft Conversion Wash Buffer to each tube of beads. Mix by pipetting up and down 15–20 times.
- 4 Place the tubes on the magnet for 30 seconds or until the solution becomes clear (up to 1 minute). Remove and discard the supernatant from each tube, then use a 20  $\mu$ L pipette to remove any residual supernatant.
- 5 Remove the tubes from the magnet and proceed immediately to [“Step 4. DNA Repair.”](#)

## Step 4. DNA Repair

- 1 Add 15  $\mu\text{L}$  of freshly prepared 0.1X Soft Conversion Elution Buffer (prepared on [page 50](#)) to each tube. With caps closed, gently flick the tubes to ensure the beads go into solution (mixing by pipetting is not needed). Spin for 1 to 2 seconds to collect the liquid at the bottom of the tubes, making sure the beads do not settle.

### NOTE

When handling multiple samples, Agilent recommends aliquoting 18  $\mu\text{L}$  of 0.1X Soft Conversion Elution Buffer into the tubes of a fresh strip tube (one tube per sample). Then, use a multichannel pipette to transfer 15  $\mu\text{L}$  of the buffer from the strip tube to the beads bound with converted library. This technique helps prevent the beads from drying out.

- 2 Program the thermal cycler to run the DNA elution thermal cycler program shown in [Table 36](#). Set the heated lid to 75°C. Load the strip tubes and run the program.

**Table 36** Thermal cycler program for DNA elution

Step	Temperature	Time
Step 1	50°C	10 minutes
Step 2	10°C	Hold

- 3 Once the thermal cycler program for DNA elution reaches the 10°C hold step, remove the strip tubes from the thermal cycler. Add 5  $\mu\text{L}$  of Soft Conversion Repair Solution to each tube (mixing is not needed). Spin for 1 to 2 seconds.
- 4 Place the tubes on the magnet for 30 seconds or until the solution becomes clear (up to 1 minute).
- 5 **Transfer all 20  $\mu\text{L}$  of supernatant from each tube of the strip tube (carefully avoiding any bead carryover) to a fresh strip tube.** Proceed to [Chapter 8](#), “Indexing PCR for Methyl Sequencing.”

## 8 Indexing PCR for Methyl Sequencing

Step 1. PCR Set Up (Methyl Sequencing) [54](#)

Step 2. Indexing PCR Program (Methyl Sequencing) [55](#)

This chapter describes the steps for the indexing PCR section of the Avida Duo workflow (section 4B in [Figure 2](#) on page 11) in which the converted libraries created from the second target capture are amplified and indexed.

This chapter uses the reagents listed in [Table 37](#).

**Table 37** Reagents for indexing PCR for methyl sequencing

Reagent	Usage Notes	Kit/Storage Location
2X Methyl Amplification Mastermix (tube with black cap or bottle)	Thaw on ice.	Avida Duo Methyl Reagent Box 1, stored at $-20^{\circ}\text{C}$
Avida Index Primer Pairs	Primer pairs are provided in either a strip or a plate. Thaw strip or plate on ice.	Stored at $-20^{\circ}\text{C}$

### NOTE

**Tip for a 1-day protocol:** You can optimize efficiency by performing the indexing PCR for the methyl sequencing samples (as described here) and the indexing PCR for the targeted sequencing samples (as described in [Chapter 5](#)) on separate thermal cyclers that are running concurrently.

## Step 1. PCR Set Up (Methyl Sequencing)

### CAUTION

Take precautions to avoid amplicon contamination during setup of the indexing PCR reactions. Review [“Important practices for preventing contamination”](#) on page 12.

- 1 Thaw the 2X Methyl Amplification Mastermix and Avida Index Primer Pairs on ice before use. Once thawed, gently vortex the 2X Methyl Amplification Mastermix at low speed, and briefly centrifuge the strip or plate of Avida Index Primer Pairs.
- 2 To each tube containing a converted library (from [step 5 on page 52](#)), add 25  $\mu\text{L}$  of 2X Methyl Amplification Mastermix to each tube. Mix by pipetting up and down 15–20 times. Briefly spin.
- 3 Add 5  $\mu\text{L}$  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 index primer pair for each sample to be sequenced in the same lane.

The tubes now contain all reagents needed for indexing PCR. [Table 38](#) summarizes the contents in each PCR reaction.

**Table 38** Indexing PCR reaction components

Reagent	Volume per PCR reaction
Converted library (from <a href="#">step 5 on page 52</a> )	20 $\mu\text{L}$
2X Methyl Amplification Mastermix	25 $\mu\text{L}$
Avida Index Primer Pair	5 $\mu\text{L}$
<b>Total</b>	<b>50 <math>\mu\text{L}</math></b>

### 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 2. Indexing PCR Program (Methyl Sequencing)

- 1 Program the thermal cycler to run the indexing PCR thermal cycler program shown in [Table 39](#). Set the heated lid to 103°C. Load the strip tubes and run the program.

**Table 39** 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
		62°C	30 seconds
		65°C	1 minute
Amplification stage 2	Variable, see <a href="#">Table 40</a> for recommendations	98°C	10 seconds
		65°C	1 minute
Final extension	1	65°C	1 minute
Final hold	1	4°C	Hold

**Table 40** 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	18 or fewer • Avida Methyl 3400 DMR Cancer Panel: 16 cycles
>50 kb to 100 kb	19
10 kb to 50 kb	20
<10 kb	21

### NOTE

The cycle number recommendations in [Table 40](#) 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 2 fewer cycles than a sample input of 10 ng.

**Indexing PCR for Methyl Sequencing****Step 2. Indexing PCR Program (Methyl Sequencing)**

- 2 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.
- 3 Proceed to [Chapter 9](#), "Library Purification and Quality Assessment" to perform library purification and quality assessment on the both the indexed libraries for methyl sequencing and the indexed libraries for targeted sequencing that were prepared earlier (see [step](#) on [page 40](#)).

**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 before proceeding to library purification. Avoid prolonged storage >72 hours.

## 9 Library Purification and Quality Assessment

Step 1. Library Purification [58](#)

Step 2. Library Quality Assessment and Quantification [59](#)

This chapter describes the steps for the library purification and quality assessment section of the Avida Duo workflow (section 5 in [Figure 2](#) on page 11). The captured libraries from both target captures are purified with AMPure XP Beads. Then, the purified, indexed libraries are quantified and analyzed for quality.

This chapter uses the reagents listed in [Table 41](#).

**Table 41** Reagents for library purification and quality assessment

Reagent	Usage Notes	Kit/Storage Location
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. Library Purification

- 1 Equilibrate the AMPure XP Beads at room temperature for 30 minutes before use.
- 2 Prepare 400  $\mu\text{L}$  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  $\mu\text{L}$  (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**

Include both the indexed libraries for targeted sequencing that were generated earlier in the workflow (see [page 40](#)) and the converted, indexed libraries for methyl sequencing that were generated in the previous chapter. 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  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{L}$  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 2. 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 42](#). Follow the instructions in the linked user guide provided for each assay.

**Table 42** 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	<a href="#">D1000 ScreenTape</a>	1 µL of sample mixed with 3 µL of D1000 sample buffer*
Agilent 5200, 5300, or 5400 Fragment Analyzer system	One of the following: <a href="#">NGS Fragment Kit (1-6000 bp)</a> <a href="#">Small Fragment Kit (1-1500 bp)</a> <a href="#">HS NGS Fragment Kit (1-6000 bp)</a> <a href="#">HS Small Fragment Kit (1-1500 bp)</a>	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 43](#) and [Table 44](#) for fragment size distribution guidelines.

To illustrate typical results, representative electropherograms generated using the TapeStation system are provided in [Figure 3](#) through [Figure 6](#). Representative electropherograms generated on the Fragment Analyzer system are provided in [Figure 7](#) through [Figure 10](#)

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

**Table 43** Qualification guidelines for targeted sequencing libraries

Input DNA type	Expected DNA fragment size peak position
cfDNA	320 bp (see <a href="#">Figure 3</a> and <a href="#">Figure 7</a> for sample electropherograms)
Fragmented gDNA*	300–450 bp (see <a href="#">Figure 4</a> and <a href="#">Figure 8</a> for sample electropherograms)

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

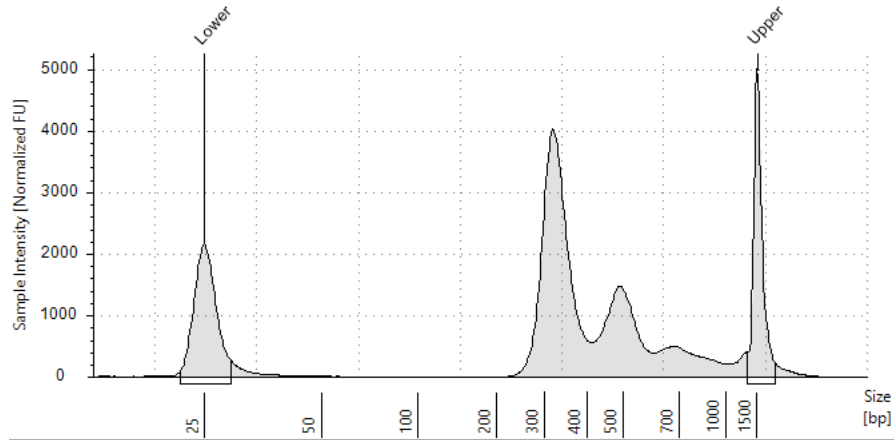
**Table 44** Qualification guidelines for methyl sequencing libraries

Input DNA type	Expected DNA fragment size peak position
cfDNA	320 bp (see <a href="#">Figure 5</a> and <a href="#">Figure 9</a> for sample electropherograms)
Fragmented gDNA*	300–350 bp (see <a href="#">Figure 6</a> and <a href="#">Figure 10</a> for sample electropherograms)

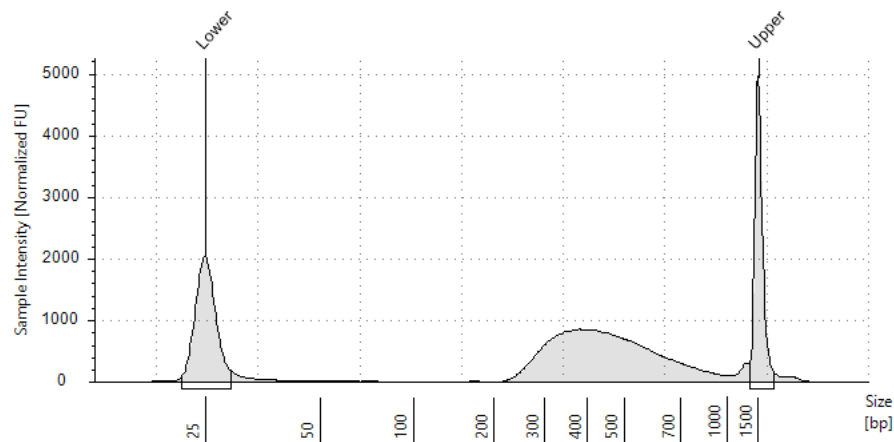
\* Refers to high-quality gDNA samples. For gDNA derived from FFPE samples, the fragment sizes may be smaller.

**Stopping Point**

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



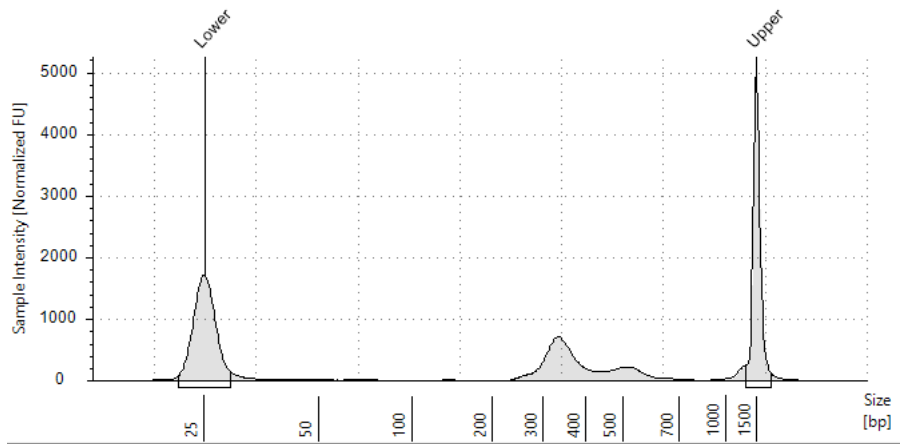
**Figure 3** Library prepared from **10 ng of a cfDNA** sample hybridized with the **Avida DNA Focused Cancer Panel** and analyzed using a D1000 ScreenTape assay on the TapeStation.



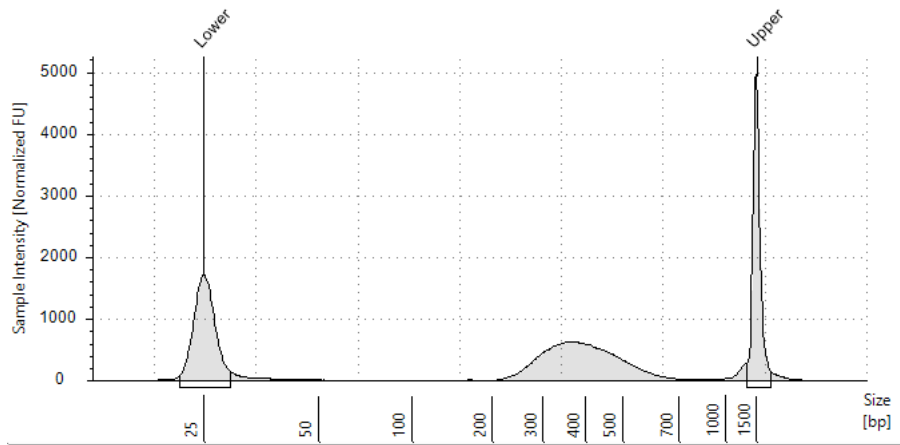
**Figure 4** Library prepared from **10 ng of a high-quality gDNA** sample hybridized with the **Avida DNA Focused Cancer Panel** and analyzed using a D1000 ScreenTape assay on the TapeStation.

## Library Purification and Quality Assessment

### Step 2. Library Quality Assessment and Quantification



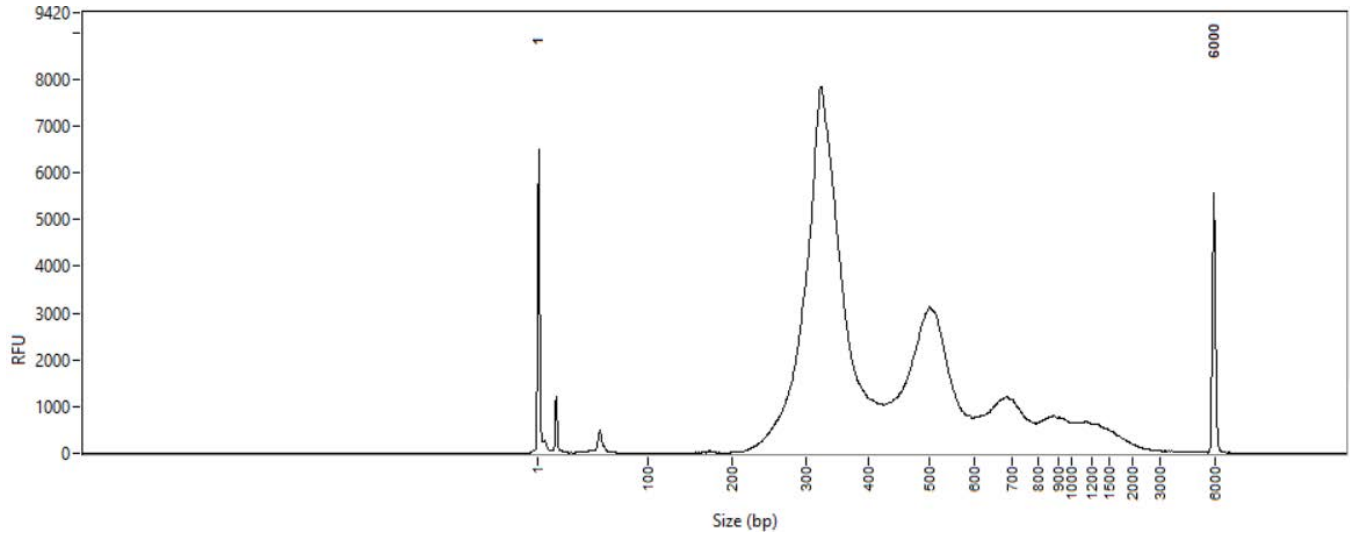
**Figure 5** Library prepared from **10 ng of a cfDNA** sample hybridized with the **Avida Methyl 3400 DMR Cancer Panel** and analyzed using a D1000 ScreenTape assay on the TapeStation.



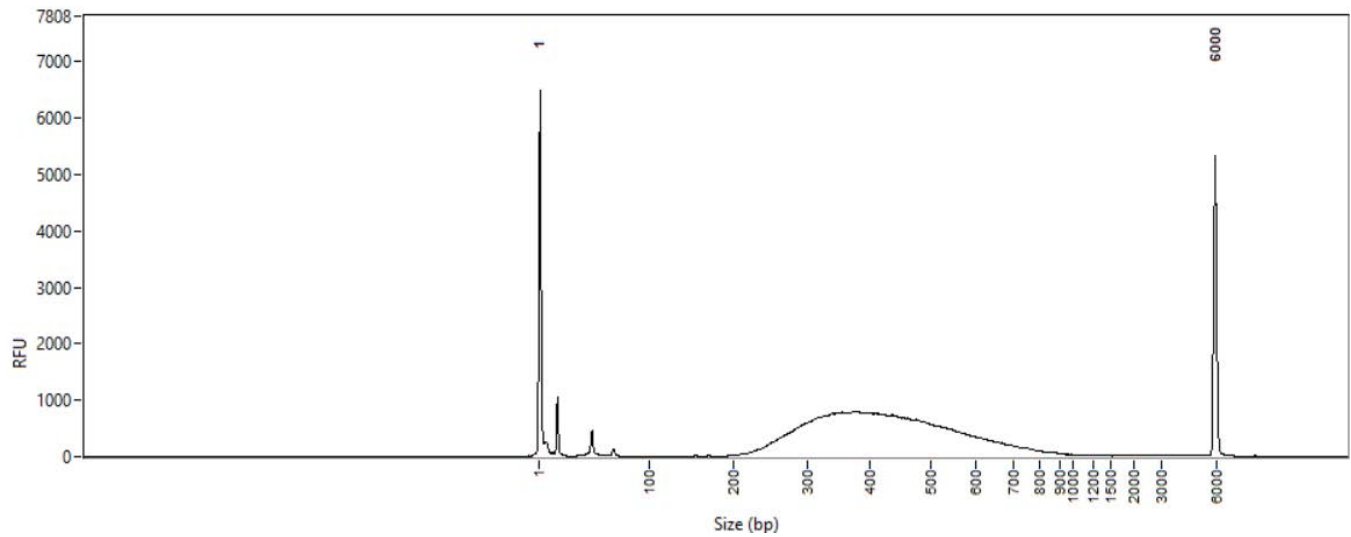
**Figure 6** Library prepared from **10 ng of a high-quality gDNA** sample hybridized with the **Avida Methyl 3400 DMR Cancer Panel** and analyzed using a D1000 ScreenTape assay on the TapeStation.

## Library Purification and Quality Assessment

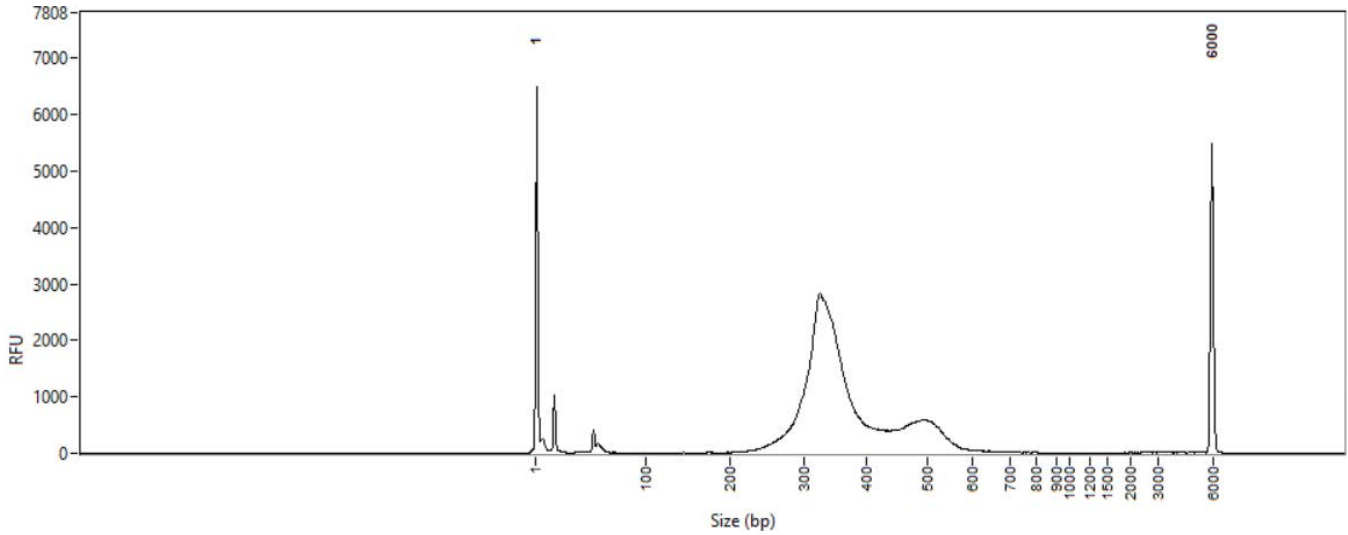
### Step 2. Library Quality Assessment and Quantification



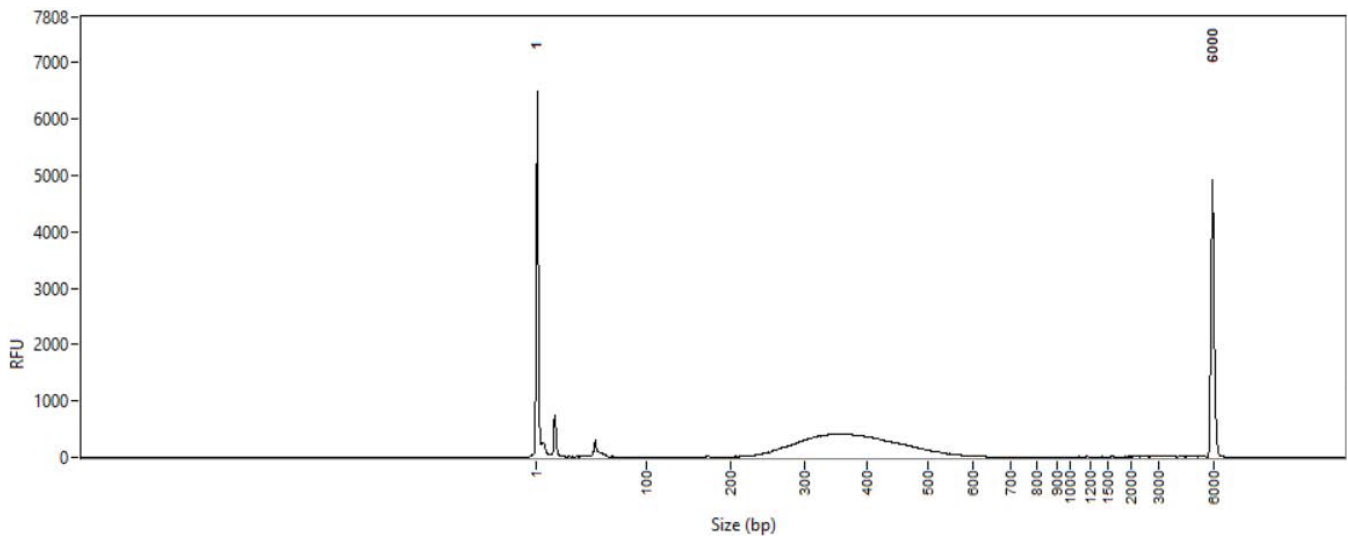
**Figure 7** Library prepared from **10 ng of a cfDNA** sample hybridized with the **Avida DNA Focused Cancer Panel** and analyzed using the NGS Fragment Kit on the Fragment Analyzer.



**Figure 8** 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.



**Figure 9** Library prepared from **10 ng of a cfDNA** sample hybridized with the **Avida Methyl 3400 DMR Cancer Panel** and analyzed using the NGS Fragment Kit on the Fragment Analyzer.



**Figure 10** Library prepared from **10 ng of a high-quality gDNA** sample hybridized with the **Avida Methyl 3400 DMR Cancer Panel** and analyzed using the NGS Fragment Kit on the Fragment Analyzer.

## 10 Sequencing and NGS Analysis

Step 1. Pool samples for multiplexed sequencing [65](#)

Step 2. Prepare the sequencing samples [66](#)

Step 3. Sequence the libraries [67](#)

Step 4. Process and analyze the reads [69](#)

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

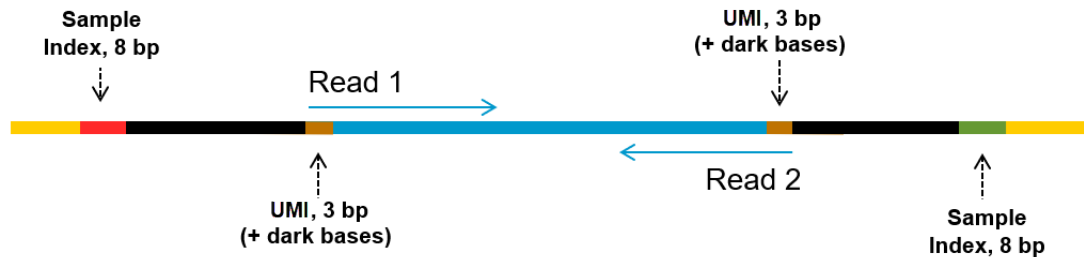
**Table 45** shows an example of the amount of 4 index-tagged samples (of different concentrations) and 1X Low TE Buffer needed for a final volume of 20  $\mu\text{L}$  at 10 nM DNA.

**Table 45** Example of volume calculation for total volume of 20  $\mu\text{L}$  at 10 nM concentration

Component	V(f)	C(i)	C(f)	#	Volume to use ( $\mu\text{L}$ )
Sample 1	20 $\mu\text{L}$	20 nM	10 nM	4	2.5
Sample 2	20 $\mu\text{L}$	10 nM	10 nM	4	5
Sample 3	20 $\mu\text{L}$	17 nM	10 nM	4	2.9
Sample 4	20 $\mu\text{L}$	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 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 3. Sequence the libraries

Proceed to cluster amplification using the appropriate Illumina Paired-End Cluster Generation Kit. [Table 46](#) 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 46](#) or provided by Illumina. Follow Illumina’s recommendation for a PhiX control in a low-concentration spike-in for improved sequencing quality control.

**Table 46** 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 47](#) showing example settings for 2 × 150 bp sequencing.

**Table 47** 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.

## Sequencing and NGS Analysis

### Step 3. Sequence the libraries

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 15 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 47](#) on page 67). 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 47](#) on page 67). 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).

### For targeted sequencing

- 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.

### For methyl sequencing

- 6 Use a suitable processing tool of your choice to trim and collect inline UMIs from each sequencing read.

- 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.
- 7 (Optional) Perform additional adaptor trimming with a tool such as Trimmomatic or Cutadapt, or hard trim both the R1 and R2 reads on the 3' end to approximately 100PE. This additional trimming may help improve the alignment rate, especially for DNA inserts that are shorter than 200 bp.
  - 8 Use Bismark or other suitable tool to align the paired end reads and generate a BAM file. An example command is below.
 

```
[path_to_bismark_command] --bowtie2 \
  [path_to_bismark_converted_reference_genome] \
  [path_to_samtools] \
  -1 R1.fq -2 R2.fq \
  --nucleotide_coverage \
  --output_dir [path_to_output_dir] \
  --temp_dir [path_to_output_dir] --basename bs_aln
```
  - 9 Remove duplicate reads based on strand, genomic location, and, optionally, UMI. For UMI based deduplication, use a tool such as Umi-Grinder. An example command is below.
 

```
[path_to_Umi-Grinder]/UmiBam -p --bam --umi --mm 1 \
  --samtools_path [path_to_samtools] [aligned_reads].bam
```
  - 10 (Optional) Analyze deduplicated reads to form duplex UMI consensus sequences.
  - 11 Collect QC metrics, such as raw coverage, deduplicated coverage, alignment percentage, and duplex rate.
  - 12 Extract CpG (5'C-phosphate-G-3') and CHX methylation information from the deduplicated reads with a tool such as *bismark\_methylation\_extractor*.  
For CHX, H is A, C or T, while X can be A, C, G or T.
  - 13 (Optional) Perform methylation profiling of CpGs in the panel regions.
  - 14 (Optional) Generate a report containing the QC metrics and methylation profile.

# 11

## Reference

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Quick Reference Protocol	79

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 14 lists the Avida Duo Methyl reagent kits and their sub-kit boxes. Detailed contents of those sub-kit boxes are shown in [Table 48](#) through [Table 52](#).

### Avida Sub-Kit Details

**Table 48** Avida Duo Methyl Reagent Box 1

Kit Component	16 Reaction Kit (p/n 5282-0151)	96 Reaction Kit (p/n 5282-0152)
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	tube with green cap
Soft Conversion Reagent A	amber tube with amber cap	tube with amber cap
2X Amplification Mastermix	tube with white cap	bottle
2X Methyl Amplification Mastermix	tube with black cap	bottle

**Table 49** 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 50** Avida Methyl and Duo Reagent Box 3

Kit Component	16 Reaction Kit (p/n 5282-0149)	96 Reaction Kit (p/n 5282-0150)
Soft Conversion Binding Beads	bottle	bottle
5X Soft Conversion Wash Buffer	bottle	bottle
Soft Conversion Elution Buffer	tube with green cap	tube with green cap
Soft Conversion Repair Solution	tube with yellow cap	tube with yellow cap
Nuclease-Free Water	tube with clear cap	bottle

**Reference**  
Avida Sub-Kit Details

**Table 51** Soft Conversion Reagent B

<b>Kit Component</b>	<b>16 Reaction Kit (p/n 5280-0188)</b>	<b>96 Reaction Kit (p/n 5280-0189)</b>
Soft Conversion Reagent B*	amber tube with blue cap	tube with blue cap

\* Soft Conversion Reagent B may be shipped as part of the Avida Methyl and Duo Reagent Box 3 or it may be shipped in its own separate bag at room temperature.

**Table 52** Avida Duo Beads Box

<b>Kit Component</b>	<b>16 Reaction Kit (p/n 5282-0153)</b>	<b>96 Reaction Kit (p/n 5282-0154)</b>
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 53](#)). 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.

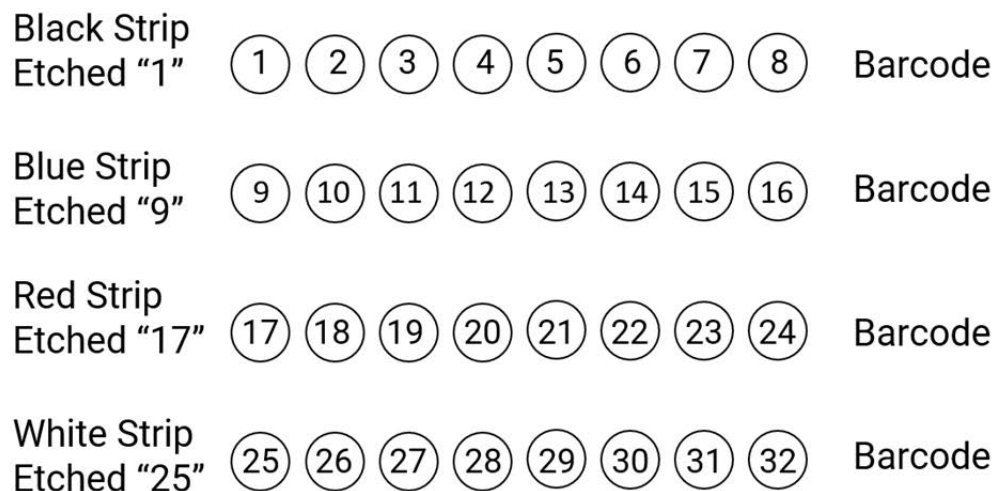
See [“Online resource for Avida index sequences”](#) on page 15 for information on downloading the Avida index sequences spreadsheet.

**Table 53** 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 Blue 8-well strip tube with index pairs 9-16 Red 8-well strip tube with index pairs 17-24 White 8-well strip tube with index pairs 25-32	Clear 96-well plate with index pairs 1–96 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 12** Map of the Avida Index Primer Pairs strip tubes provided with 16 reaction kits

[Table 54](#) and [Table 55](#) show the plate positions of the Avida Index Primer Pairs provided with 96 reaction kits.

**Table 54 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 55 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 or Avida Methyl 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 25 kb in size.

### For an Avida DNA Custom AddOn Panel

During preparation of the Hyb Mix 1 on [page 27](#), substitute the reagents and volumes in [Table 16](#) with those shown in [Table 56](#) below.

**Table 56** 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
<b>Total</b>	<b>24 µL</b>	<b>216 µL</b>	<b>432 µL</b>

### For an Avida Methyl Custom AddOn Panel

During preparation of the Methyl Hyb Mix on [page 42](#), substitute the reagents and volumes in [Table 28](#) with those shown in [Table 57](#) below.

**Table 57** Preparation of Methyl Hyb Mix using Avida Methyl Custom AddOn Panel

Reagent	Volume for 1 reaction	Volume for 8 reactions (includes excess)	Volume for 16 reactions (includes excess)
Avida Methyl 3400 DMR Cancer Panel (catalog)	4.8 µL	43.2 µL	86.4 µL
Avida Methyl Custom AddOn Panel	4.8 µL	43.2 µL	86.4 µL
Hyb Enhancer	1.5 µL	13.5 µL	27 µL
<b>Total</b>	<b>11.1 µL</b>	<b>99.9 µL</b>	<b>199.8 µL</b>

## 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 27) have equilibrated to room temperature before use.
- During indexing PCR the number of cycles used in for Amplification stage 2 may require optimization. Refer to [Table 26](#) on page 39 or [Table 40](#) on page 55, and the accompanying Note below the table, 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 12.

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

- During [“Step 3. Library bead binding”](#) on page 27, include the optional second wash (see [step 10](#) on [page 28](#)) 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 for First Target Capture”](#) and [“Step 4. Washing for Second Target Capture”](#)). 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 20](#) on page 31 and [Table 29](#) on page 42. Additionally, make sure that the thermal cycler performs the washing incubation at 60°C as directed.
- When preparing Hyb Mix 2, make sure to use the correct formulation for your panel size. [Table 18](#) on page 30 lists the formulation for Avida DNA Expanded Panel, Focused Panel, and Lymphoma Panel. [Table 19](#) on page 30 lists the formulation for Avida DNA Discovery Panel, Onco LB Panel, and Onco LB Plus Panel.

**If conversion rate is low**

- During “[Step 1. Soft Conversion](#)” on page 48, all residual supernatant must be removed from the beads before addition of the Soft Conversion Elution Buffer. 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.
- During “[Step 1. Soft Conversion](#)” on page 48, the samples need to be well mixed with Soft Conversion Mix. Make sure to pipette the mixture up and down at least 15–20 times.
- The thermal cycler program for the soft conversion reactions is provided in [Table 34](#) on page 49. Use the durations and temperatures as listed in the table.
- During “[Step 3. Washing \(Bead-Bound Converted Libraries\)](#)” on page 51, make sure that all residual 1X Soft Conversion Wash Buffer is removed from the bead pellet before adding the 0.1X Soft Conversion Elution Buffer. Refer to techniques above for collecting residual supernatant based on magnet type.

## 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/G9439-90500.pdf>.

Step	Summary of Conditions
<i>Before starting a protocol:</i> 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
<b>PCR-free Library Prep</b>	
Prepare End Prep Mix	<b>Per 8 reactions:</b> 63 µL End Prep Buffer + 27 µL End Prep Enzyme <b>Per 16 reactions:</b> 126 µL End Prep Buffer + 54 µL End Prep Enzyme Keep on ice or use immediately
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	<b>Per 8 reactions:</b> 225 µL Ligation Buffer + 54 µL Ligation Enzyme <b>Per 16 reactions:</b> 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	<b>Library Binding Beads:</b> Room temperature for at least 15 minutes <b>Hyb Blocker and Hyb Enhancer:</b> Thaw then keep at room temperature <b>Avida Panels:</b> Thaw then keep on ice <b>Hyb Buffer:</b> 37°C for at least 10–20 minutes, then keep at room temperature
Bind to Library Binding Beads	Vortex Library Binding Beads Add 87 µL Library Binding Beads to each sample Mix and spin without letting beads settle Incubate 10 min @ room temperature
<i>During 10-min incubation:</i> Prepare Hyb Mix 1	<b>Per 8 reactions:</b> 157.5 µL Nuclease-free Water + 36 µL Avida DNA Panel + 22.5 µL Hyb Blocker <b>Per 16 reactions:</b> 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
<b>First Target Capture for Targeted Sequencing</b>	
Add Hyb Mix 1	Add 24 µL Hyb Mix 1 Mix and spin

Step	Summary of Conditions
Prepare Hyb Mix 2	<p><u>Expanded, Focused, and Lymphoma panels</u>  <b>Per 8 reactions:</b> 270 µL Hyb Buffer + 54 µL Hyb Enhancer  <b>Per 16 reactions:</b> 540 µL Hyb Buffer + 108 µL Hyb Enhancer</p> <p><u>Discovery, Onco LB, and Onco LB Plus panels</u>  <b>Per 8 reactions:</b> 279 µL Hyb Buffer + 45 µL Hyb Enhancer  <b>Per 16 reactions:</b> 558 µL Hyb Buffer + 90 µL Hyb Enhancer</p>
Hybridize	<p>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</p>
<i>During hybridization program:</i> Prepare Capture Beads	<p>Mix Capture Beads well  Calculate volume of Capture Beads needed (8 µL/sample + overage)  Transfer that volume into 1.5-mL tube  Place tube on magnet, discard supernatant, then remove tube from magnet  Wash beads 2x in 100 µL preheated Hyb Wash Buffer 1, discard supernatant after each wash  (If processing &gt;16 reactions, scale the volume of Hyb Wash Buffer 1 to use 100 µL per 16 reactions; see <a href="#">Table 21</a> on page 32)  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</p>
Bead capture	<p>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  <b>Transfer all 68 µL of each supernatant to a fresh strip tube;</b> set aside for 2nd target capture  Remove samples from magnet</p>
Wash	<p>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  Add 150 µL Hyb Wash Buffer 2, and mix  Place tubes on magnet and discard supernatant  Add 20 µL Resuspension Buffer  Gently mix and spin</p>
<b>Indexing PCR for Targeted Sequencing</b>	
Set up PCR reactions	<p>Add 25 µL 2X Amplification Mastermix  Add 5 µL primer pair  Mix and spin</p>
Perform indexing PCR	<p>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 <a href="#">Table 26</a>): 10 sec @98°C, 1 min @72°C  1 min @72°C  Hold @4°C</p>

Step	Summary of Conditions
<b>Second Target Capture for Methyl Sequencing</b>	
Prepare Methyl Hyb Mix	<b>Per 8 reactions:</b> 43.2 µL Avida Methyl Panel + 13.5 µL Hyb Enhancer <b>Per 16 reactions:</b> 86.4 µL Avida Methyl Panel + 27 µL Hyb Enhancer
Hybridize	Add 6.3 µL Methyl Hyb Mix Mix and spin 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
<i>During hybridization program:</i> Prepare Capture Beads	Mix Capture Beads well Calculate volume of Capture Beads needed (8 µL/sample + overage) Transfer that volume into 1.5-mL tube 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 <a href="#">Table 30</a> on page 43) Remove tube from magnet Resuspend Capture Beads in Hyb Buffer at original volume calculated above, and mix
Bead capture	After hybridization, spin tubes Add 8 µL 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 and 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 Add 150 µL Hyb Wash Buffer 2, and mix
<b>Soft Conversion and Repair</b>	
Prep samples for conversion	Briefly spin tubes Place tubes on magnet and discard supernatant Remove ALL remaining supernatant Remove samples from magnet Add 11.5 µL Soft Conversion Elution Buffer, and pulse vortex to mix Incubate 10 min @ room temperature
<i>During 10-min incubation:</i> Prepare Soft Conversion Mix	Make sure Soft Conversion Reagent A and Soft Conversion Reagent B are well mixed <b>Per 8 reactions:</b> 31.5 µL Soft Conversion Reagent A + 180 µL Soft Conversion Reagent B <b>Per 16 reactions:</b> 63 µL Soft Conversion Reagent A + 360 µL Soft Conversion Reagent B Mix and spin
Perform soft conversion	After the 10-minute incubation, place tubes on magnet Transfer supernatant to fresh tubes Add 23.5 µL of freshly prepared Soft Conversion Mix Mix and spin Incubate in thermal cycler (heated lid 85°C): 25 min @ 75°C, Hold @ 10°C Proceed to next step as soon as thermal cycler reaches 10°C hold

Step	Summary of Conditions
Set up bead purification	Add 150 µL of Soft Conversion Binding Beads Mix and spin without letting beads settle Incubate 10 min @ room temperature
<i>During 10-min incubation:</i> Prepare 0.1X Soft Conversion Elution Buffer	<b>Per 8 reactions:</b> 16.5 µL Soft Conversion Elution Buffer + 148.5 µL Nuclease-Free Water <b>Per 16 reactions:</b> 33 µL Soft Conversion Elution Buffer + 297 µL Nuclease-Free Water Mix and spin
Wash	After the 10-minute incubation, place tubes on magnet and discard supernatant Add 150 µL of 1X Soft Conversion Wash Buffer and mix Transfer contents to fresh tubes Place tubes on magnet and discard supernatant Add 150 µL of 1X Soft Conversion Wash Buffer and mix Place tubes on magnet and discard supernatant Remove tubes from magnet
Repair	Add 15 µL of freshly prepared 0.1X Soft Conversion Elution Buffer Mix by flicking the tubes then spin without letting beads settle Incubate in thermal cycler (heated lid 75°C): 10 min @ 50°C, Hold @ 10°C As soon as thermal cycler reaches 10°C hold, add 5 µL of Soft Conversion Repair Solution Spin (without mixing) Place tubes on magnet Transfer supernatant to fresh tubes
<b>Indexing PCR for Methyl Sequencing</b>	
Set up PCR reactions	Add 25 µL 2X Methyl 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 @ 62°C, 1 minute @65°C Additional cycles (as described in <a href="#">Table 40</a> ): 10 sec @98°C, 1 min @65°C 1 min @65°C Hold @4°C
<b>Library Purification and Quality Assessment</b>	
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 2x 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)

## In This Book

This guide provides instructions for using Avida Duo Methyl Reagent Kits for library preparation, target captures, and conversion.

