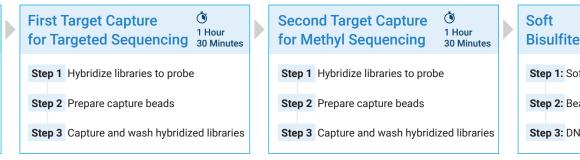
# Quick Start Protocol Avida Duo Methyl

G9439-90500 Rev B0

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











Librai Purifi	y cation	ैं 30 Minutes
Step 1	Clean-up	
Step 2	Quality control/Pool libr	aries

# This Quick Start Protocol provides key protocol details for experienced users. Visit Avida Duo Methyl Reagent Kits Protocol for the full User Guide with further information including:

- Safety information
- Notices to purchaser
- List of required materials
- QC output examples

- Critical factors for system performance including component handling, mixing, and spinning specifications; pay special attention to the key critical factors such as mixing steps highlighted in this Quick Start Protocol
- Full protocol details including Notes and Caution statements
- NGS support

## PCR-free Library Preparation

Note: Reagent vial cap colors are indicated by colored circles adjacent to reagent use instructions. Clear cap is indicated by "c" and white cap is indicated by "w".

e in next step.	Buller i Stock Dottle a	at 50 C in water b	ath or heat block until	Step 2: Ligate UN	II-tagged ada	ptors	<b>()</b> 30 m
ep 1: End repair a	nd dA-tail frag	gments	🚺 1 hour	Vortex thawed Ligati	on Buffer.		
Vortex thawed End Pre	ep Buffer at RT till a	II particles are o	dissolved.	Prepare Ligation Ma spin > keep on ice.	ster Mix at RT (Lig	MM, see Table 3	). Vortex > briefly
Add appropriate quant	tity of DNA sample	in strip tube. M	ake volume up to	Table 3: Lig MM reager	nt volumes (including	excess for suppo	rted run sizes).
50 µL with nuclease-fr	ee water > keep on	ice.	·	Reagent	Per Rxn	8 Rxn	16 Rxn
Sample quantity ran	ge:			Ligation Buffer	25 µL	225 µL	450 μL
	•			Ligation Enzyme	6 µL	54 µL	108 µL
Prepare End Prep Mix	at RT (see Table 1)	. Gently vortex a	at low speed >	Total	31 µL	279 µL	558 μL
brief spin. The End Prep Mix is st	able for up to 2 ho	urs at 4°C.		Reagents are viscou	s. Take care when J	pipetting.	
Table 1: End Prep Mix re	agent volumes (inclu	ding excess for s	upported run sizes).	📄 🗕 Add 5 µL of Adapt	or for ILM to each I	DNA sample.	
Reagent	Per Rxn	8 Rxn	16 Rxn	Do NOT add Adaptor	for ILM to the Lig	MM. Avoid expos	sing the Adaptor
End Prep Buffer	7 μL	63 µL	126 µL	to RT conditions.			- · ·
End Prep Enzyme	3 µL	27 µL	54 µL				
Total	10 µL	90 µL	180 µL	Add 31 µL of Lig MN	l to each sample. N	/lix > briefly spin.	
		·	riefly spin > keep on ice.	<ul><li>Program the therma</li><li>run the program.</li></ul>			Load the strip tube
Program the thermal of		heated lid set	to 75°C. Load the strip	Table 4: Thermal cycle	program for ligation		
tubes > run the progra	m.			Step	Tempe	rature	Time
Table 2: Thermal cycler p	program for end prep			Step 1	20°C		30 min
Step	Tempera	ture	Time	Step 2	4°C		Hold
Step 1	20°C		30 min		an in frame aval-	keen at DT	
Step 2	65°C		30 min	At 4°C Hold step, rer	nove from cycler >	keep at RT.	
Step 3	4°C		Hold				
	ove from cycler <b>&gt;</b> k			Prep Ahead: Put @ Library E Heat © Hyb Buffer to 37°C f			

### Step 3: Library bead binding



Thoroughly vortex @Library Binding Beads. Allow to settle for 5 min.

Add 87  $\mu$ L (~0.9X volume) of Library Binding Beads to each adaptor-ligated DNA sample, bringing sample volume to 183  $\mu$ L. Mix > briefly spin. Incubate at RT 10 min.

Bubbles can compromise library binding efficiency. Pipette slowly.

During 10-min incubation: Prepare Hyb Mix 1 (see Table 5). Vortex > briefly spin > keep at RT till needed.

Table 5: Hyb Mix 1 reagent volumes (including excess for supported run sizes).

		Reagent	Per Rxn	8 Rxn	16 Rxn
	©	Nuclease-Free Water	17.5 µL	157.5 μL	315 µL
		Avida DNA Panel	4 µL	36 µL	72 µL
	•	Hyb Blocker	2.5 μL	22.5 µL	45 µL
		Total	24 µL	216 µL	432 µL
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Collect beads using magnet (~2 min) > remove and discard supernatant.

Wash 1× or 2× (optional) using Library Wash Buffer. For each wash:

- □ Add 180 µL of Library Wash Buffer to each tube without disturbing the bead pellet.
- □ Incubate at RT 2 min > Remove and discard all supernatant.
   Washes completed: □ 1 □ 2
- Remove tubes from magnet.

#### Proceed immediately to First Target Capture.

## First Target Capture for Targeted Sequencing

## Step 1: Hybridize libraries to probe



Add 24 µL of Hyb Mix 1 to the beads bound with adaptor-ligated library generated in the last step. Resuspend beads by gentle vortexing or pipetting > Briefly spin.

Thoroughly vortex the Hyb Buffer that has been kept at 37°C. Keep at RT until later use.

Prepare Hyb Mix 2 based on Avida DNA panel size (see Table 6). Vortex > briefly spin > keep at RT.

Table 6: Hyb Mix 2 reagent volumes for panels <500kb and  $\ge$ 500kb (including excess for supported run sizes).

	Reagent	Per	Rxn	8 R)	kn	16 R	xn
		<500kb	≥500kb	<500kb	≥500kb	<500kb	≥500kb
©	) Hyb Buffer	30 µL	31 µL	270 µL	279 µL	540 µL	558 µL
0	Hyb Enhancer	6 µL	5 µL	54 µL	45 µL	108 µL	90 µL
	Total	36 µL	36 µL	324 µL	324 µL	648 µL	648 µL

Add 36  $\mu$ L of Hyb Mix 2 to each bead mix containing Hyb Mix 1. Mix > spin.

Program the thermal cycler (Table 7) with heated lid set to  $103^{\circ}$ C. Load the strip tubes > run the program.

Table 7: Thermal cycler program for hybridization.

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

For panels >500 kb, a longer hybridization time (up to 16 hours) may improve capture efficiency.

Step 2: Prepare	capture bead	ls (concur	rrently) 🐧 15	5 min	Step 3 continued
<ul> <li>Thoroughly pipeti</li> <li>Calculate volume Transfer into 1.5- Volume calculate</li> <li>Place tube on ma</li> <li>Wash beads 2× ir Table 8: Wash volut</li> <li># of rxns: 1-16 Volume: 100 µL</li> <li>For each wash:         <ul> <li>Add required briefly spin &gt; Washes corr</li> <li>Resuspend Captu &gt; mix well.</li> </ul> </li> </ul>	of Capture Beads mL tube. d: gnet (1 min) > disc 1preheated Hyb V me of Hyb Wash But 17-32 33-48 200 µL 300 µL volume of the pre place tube on ma ppleted:12 tre Beads in Hyb B	ture Beads st needed (8 µl card superna Wash Buffer <sup>-1</sup> ffer 1 based or 49-64 6 400 µL 5 Pheated Hyb \ gnet (1 min) 2 uffer at origin	ept at RT. tock till solution is ho L/sample + overage). tant > remove from n 1 using volume in Tak n number of reactions. 5-80 81-96 00 μL 600 μL Wash Buffer 1 to bea > discard supernatar nal volume calculated ibe of a fresh strip tul	 nagnet. ble 8. ds. Vortex > nt. d above	<ul> <li>Incubate in thermal cycler held @60°C (heated lid 75°C) for 10 minutes.</li> <li>After 10 minutes @60°C, remove tubes &gt; place on magnet (30 sec). Leave the thermal cycler running.</li> <li>Transfer all 68 µL of each supernatant to a fresh strip tube &gt; Set aside for 2nd target capture.</li> <li>Store at 4°C for up to 2 hours or -20°C overnight.</li> <li>Remove tubes containing Capture beads/library DNA hybrids from magnet. Do NOT vortex or spin the samples until instructed to do so. Handle carefully to prevent any splashing.</li> <li>Add 150 µL preheated Hyb Wash Buffer 1 to each tube. Mix well.</li> <li>Place tubes on magnet (30 sec) &gt; discard supernatant &gt; remove from magnet.</li> <li>Add 100 µL Hyb Wash Buffer 1 to each tube &gt; mix well.</li> <li>Transfer each sample to a fresh strip tube and cap tubes.</li> <li>Incubate in thermal cycler held @60°C (heated lid 75°C) for 3 minutes.</li> <li>After 3 minutes, stop the program &gt; place tubes on magnet (30 sec) &gt; discard supernatant &gt; remove from magnet.</li> <li>Add 150 µL Hyb Wash Buffer 2 kept at RT. Mix &gt; briefly spin.</li> </ul>
	n, spin tubes and p cycler for washing cler program for wa	blace on mag g (Table 9) wi	$\bigcirc$	<b>30 min</b> 5°C.	Samples can be stored in Hyb Wash Buffer 2 at 4°C overnight.         Do NOT discard the beads.         □         Place tubes on magnet (1 min) > discard all supernatant > remove tubes from magnet.         □         □       Resuspend tubes in 20 µL ● Resuspension Buffer. Vortex > briefly spin.

Transfer each supernatant to tubes containing 8 µL aliquots of prepared Capture Beads > Mix and spin.

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## Indexing PCR for Targeted Sequencing

## Step 1: Amplify and dual-index

Thaw @2X Amplification Mastermix and Avida Index Primer Pairs on ice.

Vortex 2X Amplification Mastermix at low speed. Briefly centrifuge Avida Index Primer Pairs.

Set up Indexing PCR reaction: 20  $\mu$ L resuspended Capture Beads + 25  $\mu$ L 2X Amplification Mastermix + 5  $\mu$ L of the appropriate Avida Index Primer Pair. Mix > briefly spin.

Program thermal cycler for indexing PCR (Table 10) with heated lid set to 103°C. Load the strip tubes > run the program.

Table 10: Thermal cycler program for indexing PCR

Step	Number of Cycles	Temperature	Time
Initial denaturation	1	98 °C	45 sec
Amplification stage 1	5	98 °C	10 sec
		63 °C	30 sec
		72 °C	30 sec
Amplification stage 2 based on sample input of 10 ng cfDNA	<ul> <li>11 for Discovery Cancer panel</li> <li>14 for Expanded Cancer panel</li> </ul>	98 °C	10 sec
	<ul> <li>16 or fewer for &gt;100 kb</li> <li>17 for &gt;50 kb to 100 kb</li> <li>18 for 10 kb to 50 kb (Focused Cancer panel)</li> </ul>	72 °C	1 min
	☐ 19 for <10 kb		
Final extension	1	72 °C	1 min
Final hold	1	4 °C	Hold

□ 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.

□ A sample input >50 ng typically requires 1–2 fewer cycles than a sample input of 10 ng.

At 4 °C Hold step, remove from cycler > keep on ice.

Stopping point

1 hour

Set aside indexed libraries for targeted sequencing until needed for purification and quality control steps. Store at 4°C overnight or -20°C for up to 72 hours.

## Second Target Capture for Methyl Sequencing

### Step 1: Hybridize libraries to probe



Prepare Methyl Hyb Mix (see Table 11). Vortex > briefly spin.

Table 11: Methyl Hyb Mix reagent volumes (including excess for supported run sizes).

Rea	gent	Per Rxn	8 Rxn	16 Rxn
	la Methyl 3400 DMR cer Panel	4.8 µL	43.2 µL	86.4 µL
🔵 Hyb	Enhancer	1.5 μL	13.5 µL	27 µL
Tota	al	6.3 µL	56.7 μL	113.4 µL

Add 6.3 µL of Methyl Hyb Mix to the supernatant samples that were set aside during the first target capture. Mix > briefly spin.

Program the thermal cycler (Table 12) with heated lid set to  $103^{\circ}$ C. Load the strip tubes > run the program.

Table 12: Thermal cycler program for hybridization.

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

For panels >500 kb, a longer hybridization time (up to 16 hours) may improve capture efficiency.

#### Step 2: Prepare capture beads (concurrently) Step 3: Capture and wash hybridized libraries 15 min 30 min Thoroughly vortex Hyb Buffer that has been kept at RT. After hybridization, spin tubes . Thoroughly pipette or vortex • Capture Beads stock till solution is homogeneous. Program thermal cycler for washing (Table 14) with heated lid set to 75°C. Table 14: Thermal cycler program for washing. Calculate volume of Capture Beads needed (8 µL/sample + overage). Temperature Step Time Transfer into 1.5-mL tube. 60°C Hold Step 1 Volume calculated: Add 8 µL of prepared Capture Beads to each tube > Mix and spin. Place tube on magnet (1 min) > discard supernatant > remove from magnet. Incubate in thermal cycler held at 60°C (heated lid 75°C) for 10 minutes. Wash beads 2× in 1preheated Hyb Wash Buffer 1 using volume in Table 13. After 10 minutes at 60°C, remove tubes > place on magnet (30 sec) > discard Table 13: Wash volume of Hyb Wash Buffer 1 based on number of reactions. supernatant > remove from magnet. Leave the thermal cycler running. # of rxns: 1-16 17-32 33-48 49-64 65-80 81-96 Do NOT vortex or spin the samples until instructed to do so. Handle carefully to 100 µL 200 µL 300 µL 400 µL 500 µL 600 µL Volume: prevent any splashing. For each wash: Add 150 µL preheated Hyb Wash Buffer 1 to each tube. Mix well. □ Add required volume of the preheated Hyb Wash Buffer 1 to beads. Vortex > briefly spin > place tube on magnet (1 min) > discard supernatant. Place tubes on magnet (30 sec) > discard supernatant > remove from magnet. Washes completed: 1 2 Add 100 µL Hyb Wash Buffer 1 to each tube > mix well. Resuspend Capture Beads in Hyb Buffer at original volume calculated above Transfer each sample to a fresh strip tube and cap tubes. > mix well. Incubate in thermal cycler held at 60°C (heated lid 75°C) for 3 minutes. After 3 minutes, stop the program > place tubes on magnet (30 sec) >

discard supernatant > remove samples from magnet.

Do NOT discard the beads.

Add 150 µL Hyb Wash Buffer 2 kept at RT. Mix > briefly spin.

Samples can be stored in Hyb Wash Buffer 2 at 4°C overnight.

Stopping point

## Soft Bisulfite Conversion

#### Step 1: Soft bisulfite conversion 45 min Collect beads on magnet (1 min) > remove and discard supernatant. Remove all remaining supernatant > immediately remove samples from magnet. Removing all residual Hyb Wash Buffer 2 from the beads is critical. Residual buffer may lower the bisulfite 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. Add 11.5 µL ● Soft Conversion Elution Buffer > pulse vortex > briefly spin.. Incubate 10 min at RT. During 10-minute incubation: Prepare Soft Bisulfite Conversion Mix (Table 15). Thoroughly vortex Soft Conversion Reagent A and Soft Conversion Reagent B before use > add to the mix > mix and spin. Table 15: Soft Bisulfite Conversion Mix reagent volumes (including excess for supported run sizes) Per Rxn 8 Rxn 16 Rxn Reagent Soft Conversion Reagent A 3.5 µL 31.5 µL 63 µL 20 µL Soft Conversion Reagent B 180 µL 360 µL Total 23.5 µL 211.5 µL 423 µL After the 10-minute incubation, collect beads on magnet (1 min). Transfer all supernatant to fresh tubes. Add 23.5 µL of freshly prepared Soft Bisulfite Conversion Mix > mix and spin > immediately close cap. Program the thermal cycler to run the soft bisulfite conversion (Table 16) with heated lid set to 85°C. Load strip tubes > run the program. Table 16: Thermal cycler program for soft bisulfite conversion Step Temperature Time Step 1 75°C 25 min\* 10°C Step 2 Hold \*If conversion >99.5% is desired for your assay, increase the duration of Step 1 from 25 minutes to 35 minutes. Note that the added duration will reduce molecular recovery by 10-15%. Proceed to next step as soon as thermal cycler reaches 10°C hold. Prolonged holding at 10°C may lower the recovery yield.

## Step 2: Bead purification and wash



] Thoroughly vortex and add 150  $\mu L$  of Soft Conversion Binding Beads to each tube. Mix and spin.

Incubate 10 min at RT.

During 10-minute incubation: Prepare a 0.1X dilution of Soft Conversion Elution Buffer (Table 17) for use in DNA repair step > mix and spin. Table 17: 0.1X Soft Conversion Elution Buffer volumes (including excess for supported run sizes)

	Reagent	Per Rxn	8 Rxn	16 Rxn		
	Soft Conversion Elution Buffer	1.5 μL	13.5 µL	27 µL		
©	Nuclease-Free Water	13.5 µL	121.5 µL	243 µL		
	Total	15 µL	135 µL	270 µL		

After the 10-minute incubation, collect beads on magnet (1 min). Discard all supernatant > remove tubes from magnet.

Prepare 1X stock of Soft Conversion Wash Buffer by adding 100% ethanol to 5X stock.

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

Add 150  $\mu L$  of 1X Soft Conversion Wash Buffer to each tube > mix.

Transfer contents to fresh tubes.

Collect beads on magnet (1 min) > discard all supernatant > remove from magnet.

Add 150  $\mu L$  of 1X Soft Conversion Wash Buffer to each tube > mix well

Collect beads on magnet (1 min) > remove and discard all supernatant > remove from magnet.

Proceed immediately to DNA Repair.

tep 3: DNA repai	r	<b>(</b> 15 min	In	dexii
	r prepared 0.1X Soft Conversion E ose caps > mix by flicking the tub		1	Step 1
Program thermal cy Load strip tubes > r	cler for DNA elution (Table 18) wi un the program.	ith heated lid set to 75°	C.	Thay ice.
Table 18: Thermal cyc	ler program for DNA elution			cent
Step	Temperature	Time		Set ı
Step 1	50°C	10 min		Amp
Step 2	10°C	Hold		> bri
1	cycler reaches 10°C hold, remove Solution to each tube > briefly spir		Soft [	Prog Load
Collect beads on ma	agnet (1 min)			Table
-				Step
Transfer all 20 µL of	supernatant to fresh tubes.			Initia
				Am

## ndexing PCR for Methyl Sequencing

## Step 1: Amplify and dual-index



Thaw ● 2X Methyl Amplification Mastermix and Avida Index Primer Pairs on ice. Vortex 2X Methyl Amplification Mastermix at low speed. Briefly centrifuge Avida Index Primer Pairs.

Set up Indexing PCR reaction: 20  $\mu$ L converted library + 25  $\mu$ L 2X Methyl Amplification Mastermix + 5  $\mu$ L of the appropriate Avida Index Primer Pair. Mix > briefly spin.

Program thermal cycler for indexing PCR (Table 19) with heated lid set to 103°C. Load the strip tubes > run the program.

Table 19: Thermal cycler program for indexing PCR

Step	Number of Cycles	Temperature	Time
Initial denaturation	1	98 °C	45 sec
Amplification stage 1	5	98 °C	10 sec
		62 °C	30 sec
		65 °C	1 min
Amplification stage 2 based on sample input of 10 ng cfDNA	18 or fewer for >100 kb	98 °C	10 sec
	<ul> <li>19 for &gt;50 kb to 100 kb</li> <li>20 for 10 kb to 50 kb</li> <li>21 for &lt;10 kb</li> </ul>	65 °C	1 min
Final extension	1	65 °C	1 min
Final hold	1	4 °C	Hold

- ☐ 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.
- □ A sample input >50 ng typically requires 1-2 fewer cycles than a sample input of 10 ng.
- At 4 °C Hold step, remove from cycler > keep on ice.

Stopping point

Store indexed libraries at 4°C overnight or -20°C for up to 72 hours. Avoid prolonged storage >72 hours.

**Prep Ahead:** Put AMPure XP beads at RT for use in next step (30 min equilibration). Prepare fresh 80% ethanol (400  $\mu$ L/sample) for use in next step.

## Library Purification

For remaining steps, include indexed libraries for first targeted sequencing that were generated earlier, as well as the indexed libraries for methyl sequencing from the previous step.

## Step 1: Clean-up final libraries



- Get AMPure XP beads held at RT, vortex until homogeneous.
- Add 50 µl of AMPure XP beads to each amplified DNA well > mix well. Incubate at RT 5 min.
- Collect beads using magnet (2 min) > remove and discard supernatant.
- Wash beads 2× with 200 µL freshly-prepared 80% ethanol per wash.
  - □ Add 200 µL of 80% ethanol to beads without disturbing pellet. Incubate for 30 sec > discard supernatant.
    - Washes completed: 1 2
- Remove residual ethanol and air-dry beads for up to 3 min.
- Remove tubes from magnet. Add 23  $\mu$ L of 1X Low TE Buffer to each tube. Mix > briefly spin > incubate at RT 2 min.
- Collect beads using magnet (2 min).
- ] Transfer 20  $\mu L$  of the eluate from each tube to a new tube, being careful to avoid bead carryover.

Stopping point

Store indexed libraries at 4°C overnight or -20°C for prolonged storage.

### Step 2: Quality control





Agilent 4200 or 4150 TapeStation System with D1000 Assay

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Agilent 5200, 5300 or 5400 Fragment Analyzer System with NGS Fragment Kit (1–6000 bp) or other suitable kit

### Stopping point

Varies

#### Store indexed libraries at 4°C overnight or -20°C for prolonged storage.

### Pool libraries for multiplex NGS

Calculate the number of indexes that can be combined per lane, according to sequencer capacity and amount of sequencing data required per sample.

- Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool using one of the following methods:
- □ Dilute each sample to be pooled to the concentration of the most dilute sample using Low TE > combine equal volumes of all samples in final pool.
- Combine the appropriate volume of each sample to achieve equimolar concentration in the pool, then adjust the pool to the desired final volume using Low TE. See the assay user guide for more information on this method.



### **Run notes**

Run Date		
Operator		
Probe Name or Design ID		
Index Pairs		
Reagent Lot Information	<ul> <li>Avida Duo Methyl Reagent Box 1</li> <li>Avida DNA and Duo Reagent Box 2</li> <li>Avida Methyl and Duo Reagent Box 3</li> <li>Avida Duo Beads Box</li> <li>Avida Index Primer Pairs:</li> </ul>	
Sample Information		

Library Pooling Information

Comments

Agilent Trusted Answers

This information is subject to change without notice.