Quick Start Protocol

Avida Methyl

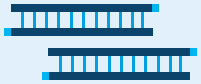
G9419-90500 Rev B0

Agilent
Trusted Answers

PCR-Free Library Preparation Workflow

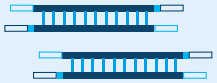
2 Hours

End repair and dA-tail fragments



1 hour

Ligate UMI-tagged adaptors



30 min

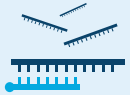
Library Bead binding

30 min

Hybridization/Capture Workflow

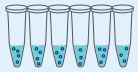
1 Hour 30 Minutes

Hybridize libraries to probe



1 hour

Prepare Capture Beads



15 min
(done concurrently)

Capture and wash hybridized libraries

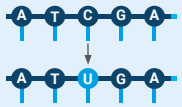
30 min

Stopping point

Soft Bisulfite Conversion/ Repair Workflow

1 Hour 30 Minutes

Soft bisulfite conversion



45 min

Bead purification and wash

30 min

DNA repair

15 min

Indexing/Library Purification Workflow

1 Hour 30 Minutes

Amplify and dual-index

1 hour

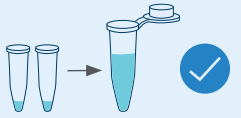
Stopping point

Clean-up final libraries

30 min

Stopping point

Quality control/ Pool libraries



Varies

Stopping point

Duration estimates are provided as guidelines for 8 reaction runs using 10 ng cfDNA. Your results may vary.

This Quick Start Protocol provides key protocol details for experienced users. Visit Avida 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".

Prep Ahead: Heat Hyb Wash Buffer 1 stock bottle at 50°C in water bath or heat block until use in next step.

Step 1: End repair and dA-tail fragments

1 hour

- [ ] Vortex thawed End Prep Buffer at RT till all particles are dissolved.
- [ ] Add appropriate quantity of DNA sample in strip tube. Make volume up to 50 µL with nuclease-free water **>** keep on ice.

  Sample quantity range: _____
- [ ] Prepare End Prep Mix at RT (see Table 1). Gently vortex at low speed **>** brief spin.

  The End Prep Mix is stable for up to 2 hours at 4°C.

  Table 1: End Prep Mix reagent volumes (including excess for supported run sizes).

Reagent	Per Rxn	8 Rxn	16 Rxn
End Prep Buffer	7 µL	63 µL	126 µL
End Prep Enzyme	3 µL	27 µL	54 µL
Total	10 µL	90 µL	180 µL

- [ ] Add 10 µL of End Prep Mix to each DNA sample. Mix **>** briefly spin **>** keep on ice.
- [ ] Program the thermal cycler (Table 2) with heated lid set to 75°C. Load the strip tubes **>** run the program.

  Table 2: Thermal cycler program for end prep.

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

- [ ] At 4°C Hold step, remove from cycler **>** keep at RT.

Prep Ahead: Thaw ● Ligation Buffer (at RT) and ● Adaptor for ILM (RT > ice) for use in next step.

Step 2: Ligate UMI-tagged adaptors

30 min

- [ ] Vortex thawed Ligation Buffer.
- [ ] Prepare Ligation Master Mix at RT (Lig MM, see Table 3). Vortex **>** briefly spin **>** keep on ice.

  Table 3: Lig MM reagent volumes (including excess for supported run sizes).

Reagent	Per Rxn	8 Rxn	16 Rxn
● Ligation Buffer	25 µL	225 µL	450 µL
● Ligation Enzyme	6 µL	54 µL	108 µL
Total	31 µL	279 µL	558 µL

  Reagents are viscous. Take care when pipetting.
- [ ] Add 5 µL of ● Adaptor for ILM to each DNA sample.

  Do NOT add Adaptor for ILM to the Lig MM. Avoid exposing the Adaptor to RT conditions.
- [ ] Add 31 µL of Lig MM to each sample. Mix **>** briefly spin.
- [ ] Program the thermal cycler (Table 4) with heated lid off. Load the strip tubes **>** run the program.

  Table 4: Thermal cycler program for ligation.

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

- [ ] At 4°C Hold step, remove from cycler **>** keep at RT.

Prep Ahead: Put Ⓦ Library Binding Beads at RT for use in next step (15 min equilibration) for use in next step. Heat Ⓒ Hyb Buffer to 37°C for use in "Target Capture" steps. Thaw ● Hyb Blocker, ● Hyb Enhancer and Avida Panel at RT for use in next steps. Transfer Avida Panel to ice once thawed.

Step 3: Library bead binding

30 min

- ☐ Thoroughly vortex Ⓦ Library Binding Beads. Allow to settle for 5 min.
- ☐ Add 87 µL (~0.9X volume) of Library Binding Beads to each adaptor-ligated DNA sample, bringing sample volume to 183 µL. Mix **>** 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 Methyl 3400 DMR Cancer Panel	4 µL	36 µL	72 µL
Hyb Blocker	2.5 µL	22.5 µL	45 µL
Total	24 µL	216 µL	432 µL

- ☐ 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 Hybridization.

Hybridization/Capture

Step 1: Hybridize libraries to probe

1 hour

- ☐ 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 (see Table 6). Vortex **>** briefly spin **>** keep at RT.

Table 6: Hyb Mix 2 reagent volumes (including excess for supported run sizes).

Reagent	Per Rxn	8 Rxn	16 Rxn
Ⓒ Hyb Buffer	30 µL	270 µL	540 µL
Hyb Enhancer	6 µL	54 µL	108 µL
Total	36 µL	324 µL	648 µL

- ☐ Add 36 µL 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°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 beads (concurrently)

15 min

- [ ] Thoroughly vortex Hyb Buffer that has been kept at RT.
- [ ] Thoroughly pipette or vortex Capture Beads stock till solution is homogeneous.
- [ ] Calculate volume of Capture Beads needed (8 µL/sample + overage). Transfer into PCR tube or 1.5-mL tube.

  Volume calculated: _____
- [ ] Place tube on magnet (1 min) **>** discard supernatant **>** remove from magnet.
- [ ] Wash beads 2× in 1preheated Hyb Wash Buffer 1 using volume in Table 8.

Table 8: Wash volume of Hyb Wash Buffer 1 based on number of reactions.

# of rxns:	1-16	17-32	33-48	49-64	65-80	81-96
Volume:	100 µL	200 µL	300 µL	400 µL	500 µL	600 µL

For each wash:

- ☐ Add required volume of the preheated Hyb Wash Buffer 1 to beads. Vortex **>** briefly spin **>** place tube on magnet (1 min) **>** discard supernatant.

  Washes completed: ☐1 ☐2

- [ ] Resuspend Capture Beads in Hyb Buffer at original volume calculated above **>** mix well.
- [ ] Add 8 µL resuspended Capture Beads to each tube of a fresh strip tube.

Step 3: Capture and wash hybridized libraries

30 min

- [ ] After hybridization, spin tubes and place on magnet (1 min).
- [ ] Program thermal cycler for washing (Table 9) with heated lid set to 75°C.

Table 9: Thermal cycler program for washing.

Step	Temperature	Time
Step 1	60°C	Hold

- [ ] Transfer each supernatant to tubes containing 8 µL aliquots of prepared Capture Beads **>** Mix and spin.
- [ ] Incubate in thermal cycler held @60°C (heated lid 75°C) for 10 minutes.
- [ ] After 10 minutes @60°C, remove tubes **>** place on magnet **>** discard supernatant **>** remove from magnet. Leave the thermal cycler running.

  Do NOT vortex or spin the samples until instructed to do so. Handle carefully to prevent any splashing.
- [ ] Add 150 µL preheated Hyb Wash Buffer 1 to each tube. Mix well.
- [ ] Place tubes on magnet (30 sec) **>** discard supernatant **>** remove from magnet.
- [ ] Add 100 µL Hyb Wash Buffer 1 to each tube **>** mix well.
- [ ] Transfer each sample to a fresh strip tube and cap tubes.
- [ ] Incubate in thermal cycler held @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.
- [ ] Add 150 µL Hyb Wash Buffer 2 kept at RT. Mix **>** briefly spin.

Stopping point

Samples can be stored in Hyb Wash Buffer 2 at 4°C overnight.
Do NOT discard the beads.

Soft Bisulfite Conversion/Repair

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 10). Thoroughly vortex Soft Conversion Reagent A and Soft Conversion Reagent B before use **>** add to the mix **>** mix and spin.

  Table 10: Soft Bisulfite Conversion Mix reagent volumes (including excess for supported run sizes)

Reagent	Per Rxn	8 Rxn	16 Rxn
● Soft Conversion Reagent A	3.5 µL	31.5 µL	63 µL
● Soft Conversion Reagent B	20 µL	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 11) with heated lid set to 85°C. Load strip tubes **>** run the program.

  Table 11: Thermal cycler program for soft bisulfite conversion

Step	Temperature	Time
Step 1	75°C	25 min*
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. 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

30 min

- [ ] Thoroughly vortex and add 150 µL 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 12) for use in DNA repair step **>** mix and spin.

  Table 12: 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 µL 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 µL of 1X Soft Conversion Wash Buffer to each tube **>** mix.
- [ ] Collect beads on magnet (1 min) **>** remove and discard all supernatant **>** remove from magnet.
- [ ] Proceed immediately to DNA Repair.

Step 3: DNA repair

15 min

- [ ] Add 15 µL of freshly prepared 0.1X Soft Conversion Elution Buffer (prepared in previous step). Close caps **>** mix by flicking the tubes **>** briefly spin.
- [ ] Program thermal cycler for DNA elution (Table 13) with heated lid set to 75°C. Load strip tubes **>** run the program.

Table 13: Thermal cycler program for DNA elution

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

- [ ] As soon as thermal cycler reaches 10°C hold, remove tubes **>** add 5 µL of ●Soft Conversion Repair Solution to each tube **>** briefly spin.
- [ ] Collect beads on magnet (1 min).
- [ ] Transfer all 20 µL of supernatant to fresh tubes.

Indexing/Library Purification

Step 1: Amplify and dual-index

1 hour

- [ ] 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 µL converted library + 25 µL 2X Methyl Amplification Mastermix + 5 µL of the appropriate Avida Index Primer Pair. Mix **>** briefly spin.
- [ ] Program thermal cycler for indexing PCR (Table 14) with heated lid set to 103°C. Load the strip tubes **>** run the program.

Table 14: 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) ☐ 16 for 3400 DMR Cancer panel ☐ 18 or fewer for >100 kb ☐ 19 for >50 kb to 100 kb ☐ 20 for 10 kb to 50 kb ☐ 21 for <10 kb		98 °C	10 sec
		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 µL/sample) for use in next step.

Step 2: Clean-up final libraries

30 min

- ☐ 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. For each 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 µL of 1X Low TE Buffer to each tube. Mix **>** briefly spin **>** incubate at RT 2 min.
- ☐ Collect beads using magnet (2 min).
- ☐ Transfer 20 µL of the eluate from each tube to a new tube, being careful to avoid bead carryover.

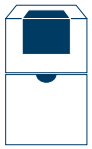
Stopping point

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

Step 3: Quality control

Varies

Assess quality and quantity using one of these platforms:



Agilent 4200 or 4150 TapeStation System with D1000 Assay

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

Stopping point

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

Step 3 continued

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	
Reagent Lot Information	– Methyl Reagent Box 1: – Methyl Reagent Box 2: – Methyl and Duo Reagent Box 3 – Probe: – Avida Beads Box: – Avida Index Primer Pairs:
Sample Information	
Library Pooling Information	
Comments	

G9419-90500 Rev B0, June 2024

Agilent
Trusted Answers