

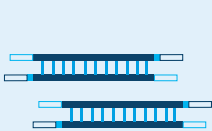



G9409-90500 Rev B1  
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## PCR-Free Library Preparation Workflow 2 Hours


End repair and dA-tail fragments 

 1 hour

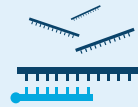
Ligate UMI-tagged adaptors 


 30 min

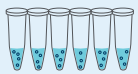
Library Bead binding 


 30 min


## Hybridization/Capture Workflow 1 Hour 30 Minutes



Hybridize libraries to probe 

 1 hour


Prepare capture beads 



 15 min  
(done concurrently with hybridization)


Capture and wash hybridized libraries 



 30 min 

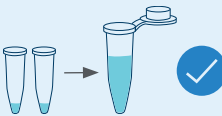
## Indexing/Library Purification Workflow 1 Hour 30 Minutes



Amplify and dual-index 


 1 hour 

Clean-up 

 30 min 

Quality control/Pool libraries 

 Varies 

 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 DNA 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
<b>Total</b>	<b>10 µL</b>	<b>90 µL</b>	<b>180 µL</b>

- 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
<b>Total</b>	<b>31 µL</b>	<b>279 µL</b>	<b>558 µL</b>

- 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 with heated lid turned off. Load the strip tubes > run the program.
- Table 4: Thermal cycler program for end prep.

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  $\mu\text{L}$  (~0.9X volume) of Library Binding Beads to each adaptor-ligated DNA sample, bringing sample volume to 183  $\mu\text{L}$ . Mix  $\blacktriangleright$  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  $\blacktriangleright$  briefly spin  $\blacktriangleright$  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 $\mu\text{L}$	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>

- Collect beads using magnet (~2 min)  $\blacktriangleright$  remove and discard supernatant.
- Wash 1 $\times$  or 2 $\times$  (optional) using Library Wash Buffer. For each wash:
  - Add 180  $\mu\text{L}$  of Library Wash Buffer to each tube without disturbing the bead pellet.
  - Incubate at RT 2 min  $\blacktriangleright$  Remove and discard all supernatant.
 Washes completed:  1  2
- Remove tubes from magnet.

Proceed immediately to Hybridization.

## Hybridization/Capture/Indexing

### Step 1: Hybridize libraries to probe

1 hour

- Add 24  $\mu\text{L}$  of Hyb Mix 1 to the beads bound with adaptor-ligated library generated in the last step. Resuspend beads by gentle vortexing or pipetting  $\blacktriangleright$  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 the covered size of the Avida DNA panel (see Table 6). Vortex  $\blacktriangleright$  briefly spin  $\blacktriangleright$  keep at RT.

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

Reagent	Per Rxn		8 Rxn		16 Rxn	
	<500kb	$\geq$ 500kb	<500kb	$\geq$ 500kb	<500kb	$\geq$ 500kb
Hyb Buffer	30 $\mu\text{L}$	31 $\mu\text{L}$	270 $\mu\text{L}$	279 $\mu\text{L}$	540 $\mu\text{L}$	558 $\mu\text{L}$
Hyb Enhancer	6 $\mu\text{L}$	5 $\mu\text{L}$	54 $\mu\text{L}$	45 $\mu\text{L}$	108 $\mu\text{L}$	90 $\mu\text{L}$
<b>Total</b>	<b>36 <math>\mu\text{L}</math></b>	<b>36 <math>\mu\text{L}</math></b>	<b>324 <math>\mu\text{L}</math></b>	<b>324 <math>\mu\text{L}</math></b>	<b>648 <math>\mu\text{L}</math></b>	<b>648 <math>\mu\text{L}</math></b>

- Add 36  $\mu\text{L}$  of Hyb Mix 2 to each bead mix containing Hyb Mix 1. Mix  $\blacktriangleright$  spin.
- Program the thermal cycler (Table 7) with heated lid set to 103°C. Load the strip tubes  $\blacktriangleright$  run the program.

Table 7: Thermal cycler program for hybridization.

Step	Temperature	Time
Step 1	98°C	2 min
Step 2	$\geq$ 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 pipett or vortex Capture Beads stock till solution is homogeneous.
- Calculate volume of Capture Beads needed (8  $\mu$ L/sample + overage). Transfer into 1.5-mL tube.  
Volume calculated: \_\_\_\_\_
- Place tube on magnet (1 min) > discard supernatant > remove from magnet.
- Wash beads 2x 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 $\mu$ L	200 $\mu$ L	300 $\mu$ L	400 $\mu$ L	500 $\mu$ L	600 $\mu$ L
- 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  $\mu$ L 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 hybridization.

Step	Temperature	Time
Step 1	60°C	Hold
- Transfer each supernatant to tubes containing 8  $\mu$ L 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  $\mu$ L preheated Hyb Wash Buffer 1 to each tube. Mix well.
- Place tubes on magnet (30 sec) > discard supernatant > remove from magnet.
- Add 100  $\mu$ L 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  $\mu$ L 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.*

- Place tubes on magnet (1 min) > discard all supernatant > remove tubes from magnet.
- Resuspend tubes in 20  $\mu$ L  Resuspension Buffer. Vortex > briefly spin.

# Indexing/Library Purification

## Step 1: Amplify and dual-index

 1 hour

- 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 µL resuspended Capture Beads + 25 µL 2X Amplification Mastermix + 5 µL 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)	<input type="checkbox"/> 11 for Discovery Cancer panel	98 °C	10 sec
	<input type="checkbox"/> 13 for Onco LB Plus panel		
	<input type="checkbox"/> 14 for Expanded Cancer panel	72 °C	1 min
	<input type="checkbox"/> 14 for Onco LB panel		
	<input type="checkbox"/> 16 for Lymphoma panel		
	<input type="checkbox"/> 16 or fewer for >100 kb		
	<input type="checkbox"/> 17 for >50 kb to 100 kb		
<input type="checkbox"/> 18 for 10 kb to 50 kb (Focused Cancer panel)			
<input type="checkbox"/> 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

*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 2x 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



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.*

### 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

- DNA Reagent Box 1:
- Avida DNA and Duo Reagent Box 2:
- Probe:
- Avida Beads Box:
- Avida Index Primer Pairs:

Sample Information

Library Pooling Information

Comments

This information is subject to change without notice.