Prepare cfDNA and Perform End Repair and A-Tailing

- ☐ 1 Bring cfDNA to room temperature.
- ☐ 2 See *cfDNA Input Recommendations* on page 1 to quantify samples.
- ☐ 3 Use RSB to prepare a minimum of 30 ng of each purified cfDNA sample in a final volume of 50 µl (0.6 ng/µl).
- ☐ 4 Pipette mix or vortex cfDNA and then centrifuge briefly.
- ☐ 5 Label the MIDI plate LP (Library Preparation).
- ☐ 6 Preheat two Hybex incubators with MIDI heat block inserts as follows.
  - Preheat the first incubator to 30°C.
  - Preheat the second incubator to 72°C.
- ☐ 7 Transfer 50 µl of each cfDNA sample to corresponding wells of a new 96-well MIDI plate.
- ☐ 8 Prepare an ice bucket.
- ☐ 9 Combine the appropriate volumes from the table below in a microcentrifuge tube to prepare ERA1 Master Mix. Please note that master mix volumes include overage.

Master Mix Component	3 Samples (µl)	24 Samples (µl)
ERA1-B	26	207
ERA1-A	10	81

- ☐ 10 Pipette 10 times to mix, and then place ERA1 Master Mix on ice.
- ☐ 11 Add 10 µl ERA1 Master Mix to each sample in the LP MIDI plate.
- ☐ 12 Shake the plate at 1800 rpm for 1 minute.
- ☐ 13 Incubate at 30°C for 30 minutes.
- ☐ 14 Immediately transfer to another incubator at 72°C and incubate for 20 minutes.
- ☐ 15 Place the LP MIDI plate on ice for 5 minutes.

Ligate Adapters

- ☐ 1 Add 60 µl ALB1.
- ☐ 2 Add 5 µl LIG3.
- ☐ 3 Add 10 µl UMI1.
- ☐ 4 Shake the plate at 1800 rpm for 1 minute.
- ☐ 5 Incubate at room temperature for 30 minutes.
- ☐ 6 Add 5 µl STL.
- ☐ 7 Shake the plate at 1800 rpm for 1 minute.

Clean Up Ligation

- ☐ 1 Label a new 96-well PCR plate LS.
- ☐ 2 Prepare fresh 80% EtOH.
- ☐ 3 Vortex SPB for 1 minute to resuspend the beads.
- ☐ 4 Add 112 µl SPB to the LP MIDI plate.
- ☐ 5 Shake the plate at 1800 rpm for 1 minute.
- ☐ 6 Incubate at room temperature for 5 minutes.
- ☐ 7 Place the LP MIDI plate on the magnetic stand for 10 minutes.
- ☐ 8 Remove and discard all supernatant.
- ☐ 9 Add 200 µl EtOH, and then remove EtOH after 30 seconds.
- ☐ 10 Repeat step 9 to wash a **second** time.
- ☐ 11 Use a P20 pipette with fine tips to remove residual supernatant.
- ☐ 12 Remove from the magnetic stand.
- ☐ 13 Add 27.5 µl RSB.
- ☐ 14 Shake the LP MIDI plate at 1800 rpm for 1 minute.
- ☐ 15 Incubate at room temperature for 2 minutes.
- ☐ 16 Place on a magnetic stand for 2 minutes.
- ☐ 17 Transfer 25 µl of each eluate from the LP MIDI plate to the LS PCR plate.

Index PCR

- ☐ 1 Assign one UPxx index primer per library (xx = index primer number).
- ☐ 2 In the post-amp area, save the following I-PCR program on the thermal cycler:
  - Choose the preheat lid option and set to 100°C
  - Set the reaction volume to 50 µl
  - 98°C for 30 seconds
  - 15 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 10°C
- ☐ 3 Add 5 µl of the assigned indexing primer (UPxx) to the LS PCR plate. Apply a new tube cap to the indexing primer tube with a cap provided in your kit.
- ☐ 4 Add 20 µl EPM.
- ☐ 5 Shake the plate at 1800 rpm for 1 minute.
- ☐ 6 Transfer the plate to the post-amp area to prevent amplification product carryover.
- ☐ 7 Briefly centrifuge at 280 × g.
- ☐ 8 Place on the thermal cycler and run the I-PCR program.
- ☐ 9 Relabel the plate ALS.
- ☐ 10 Centrifuge briefly.

SAFE STOPPING POINT

If you are stopping, make sure that the ALS PCR plate is sealed well, and store at -25°C to -15°C for up to 30 days.

Set Up First Hybridization

- ☐ 1 If the ALS PCR plate was stored at -25°C to -15°C, prepare it as follows.
  - ☐ a Thaw at room temperature.
  - ☐ b Centrifuge at 280 × g for 1 minute.
  - ☐ c Pipette to mix and centrifuge.
- ☐ 2 Label a new 96-well PCR plate HYB1.
- ☐ 3 Save the following HYB1 program on the thermal cycler:
  - Choose the preheat lid option and set to 100°C
  - Set the reaction volume to 50 µl
  - 95°C for 10 minutes
  - 85°C for 2.5 minutes
  - 75°C for 2.5 minutes
  - 65°C for 2.5 minutes
  - Hold at 57°C
- ☐ 4 Transfer 20 µl of each library to the HYB1 PCR plate.
- ☐ 5 Add 15 µl TCB1.
- ☐ 6 Add 10 µl TCA1.
- ☐ 7 Add 5 µl OPD2 .
- ☐ 8 Shake the plate at 1800 rpm for 1 minute.
- ☐ 9 Place on the thermal cycler and run the HYB1 program. Hybridize for 8–24 hours (overnight) at 57°C.

Capture Targets One

- ☐ 1 Preheat a Hybex incubator with MIDI heat block insert to 57°C.
- ☐ 2 Label a new 96-well MIDI plate CAP1 (Capture 1).
- ☐ 3 Label a new 96-well PCR plate ELU1 (Elution 1).
- ☐ 4 Remove the HYB1 PCR plate from the thermal cycler.
- ☐ 5 Vortex SMB for 1 minute to resuspend the beads.
- ☐ 6 Add 150 µl SMB to the CAP1 MIDI plate.
- ☐ 7 Transfer 50 µl from the HYB1 PCR plate to the CAP1 MIDI plate.
- ☐ 8 Shake the plate at 1800 rpm for 1 minute.
- ☐ 9 Incubate in a Hybex incubator at 57°C for 25 minutes.
- ☐ 10 Place on a magnetic stand for 2 minutes.
- ☐ 11 Remove and discard all supernatant.
- ☐ 12 Wash beads as follows.
  - ☐ a Remove the CAP1 MIDI plate from the magnetic stand.
  - ☐ b Add 200 µl EEW.
  - ☐ c Apply Microseal 'B' to the CAP1 MIDI plate and shake the CAP1 MIDI plate at 1800 rpm for 2 minutes.
  - ☐ d Incubate in a Hybex incubator at 57°C for 10 minutes.
  - ☐ e Place on a magnetic stand for 2 minutes.
  - ☐ f Use a pipette to remove and discard all supernatant.
- ☐ 13 Repeat step 12 to wash beads a **second** time.
- ☐ 14 Use a P20 pipette with fine tips to remove any residual supernatant from each well.
- ☐ 15 Combine the following volumes in a microcentrifuge tube to prepare the EE2+HP3 Elution Mix:

Elution Mix Component	3 Libraries (µl)	24 Libraries (µl)
EE2	95	513
HP3	5	27

- ☐ 16 Vortex briefly.
- ☐ 17 Remove the CAP1 MIDI plate from the magnetic stand.
- ☐ 18 Add 17 µl EE2+HP3 Elution Mix.
- ☐ 19 Shake the plate at 1800 rpm for 1 minute.
- ☐ 20 Place on a magnetic stand for 2 minutes.
- ☐ 21 Transfer 15 µl eluate from the CAP1 MIDI plate to the ELU1 PCR plate.
- ☐ 22 Add 5 µl ET2 to the ELU1 PCR plate.
- ☐ 23 Shake the plate at 1800 rpm for 1 minute.

Set Up Second Hybridization

- ☐ 1 Save the following HYB2 program on the thermal cycler:
  - Choose the preheat lid option and set to 100°C
  - Set the reaction volume to 50 µl
  - 95°C for 10 minutes
  - 85°C for 2.5 minutes
  - 75°C for 2.5 minutes
  - 65°C for 2.5 minutes
  - Hold at 57°C
- ☐ 2 Add 15 µl TCB1 to the ELU1 PCR plate.
- ☐ 3 Add 10 µl TCA1.
- ☐ 4 Add 5 µl OPD2 .
- ☐ 5 Shake the plate at 1800 rpm for 1 minute.
- ☐ 6 Place on the thermal cycler and run the HYB2 program. Hybridize at 57°C for 1.5-4 hours.

Capture Targets Two

- ☐ 1 Preheat a Hybex incubator with MIDI heat block insert to 57°C.
- ☐ 2 Remove the ELU1 PCR plate from the thermal cycler.
- ☐ 3 Vortex SMB for 1 minute to resuspend the beads.
- ☐ 4 Add 150 µl SMB to the CAP2 MIDI plate.
- ☐ 5 Transfer 50 µl from the ELU1 PCR plate to the CAP2 MIDI plate.
- ☐ 6 Shake the plate at 1800 rpm for 1 minute.
- ☐ 7 Incubate in a Hybex incubator at 57°C for 25 minutes.
- ☐ 8 Place on a magnetic stand for 2 minutes.
- ☐ 9 Remove and discard all supernatant.
- ☐ 10 Remove the CAP2 MIDI plate from the magnetic stand.
- ☐ 11 Add 200 µl RSB.
- ☐ 12 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 13 Place on a magnetic stand for 2 minutes.
- ☐ 14 Remove and discard all supernatant.
- ☐ 15 Use a P20 pipette with fine tips to remove any residual supernatant.
- ☐ 16 Combine the following volumes in a microcentrifuge tube to prepare the EE2+HP3 Elution Mix:

Elution Mix Component	3 Libraries (µl)	24 Libraries (µl)
EE2	95	627
HP3	5	33

- ☐ 17 Vortex to mix.
- ☐ 18 Remove the CAP2 MIDI plate from the magnetic stand.
- ☐ 19 Add 22 µl EE2+HP3 Elution Mix.
- ☐ 20 Shake the CAP2 MIDI plate at 1800 rpm for 1 minute.
- ☐ 21 Place on a magnetic stand for 2 minutes.
- ☐ 22 Transfer 20 µl eluate from the CAP2 MIDI plate to the ELU2 PCR plate.
- ☐ 23 Add 5 µl ET2 to the ELU2 PCR plate.
- ☐ 24 Shake the ELU2 PCR plate at 1800 rpm for 1 minute.
- ☐ 25 Centrifuge briefly.

SAFE STOPPING POINT

If you are stopping, store ELU2 PCR plate at -25°C to -15°C for up to 7 days.

Amplify Enriched Library

- ☐ 1 If the ELU2 PCR plate was stored at -25°C to -15°C, prepare it as follows.
  - ☐ a Thaw at room temperature.
  - ☐ b Centrifuge at 280 × g for 1 minute.
  - ☐ c Pipette to mix and centrifuge.
- ☐ 2 Save the following EL-PCR program on the thermal cycler:
  - Choose the preheat lid option and set to 100°C
  - Set the reaction volume to 50 µl
  - 98°C for 30 seconds
  - 18 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 10°C
- ☐ 3 Add 5 µl PPC3 to the ELU2 PCR plate.
- ☐ 4 Add 20 µl EPM.
- ☐ 5 Shake the ELU2 PCR plate at 1800 rpm for 1 minute.
- ☐ 6 Briefly centrifuge at 280 × g.
- ☐ 7 Place on the preprogrammed thermal cycler and run the EL-PCR program.

Clean Up Amplified Enriched Library

- ☐ 1 Label a new 96-well MIDI plate BIND1.
- ☐ 2 Label a new 96-well PCR plate PL (Purified Libraries).
- ☐ 3 Prepare fresh 80% EtOH.
- ☐ 4 Remove the ELU2 PCR plate from the thermal cycler and set aside.
- ☐ 5 Vortex SPB for 1 minute to resuspend the beads.
- ☐ 6 Add 110 µl SPB to the BIND1 MIDI plate.
- ☐ 7 Transfer 50 µl from the ELU2 PCR plate to the BIND1 MIDI plate.
- ☐ 8 Shake the plate at 1800 rpm for 1 minute.
- ☐ 9 Incubate at room temperature for 5 minutes.
- ☐ 10 Place the BIND1 MIDI plate on magnetic stand for 5 minutes.
- ☐ 11 Remove and discard all supernatant.
- ☐ 12 Add 200 µl EtOH, and then remove EtOH after 30 seconds.
- ☐ 13 Repeat step 12 to wash a **second** time.
- ☐ 14 Use a P20 pipette with fine tips to remove residual supernatant.
- ☐ 15 Remove the BIND1 MIDI plate from the magnetic stand.
- ☐ 16 Add 32 µl RSB.
- ☐ 17 Shake the plate at 1800 rpm for 1 minute.
- ☐ 18 Incubate at room temperature for 2 minutes.
- ☐ 19 Place on a magnetic stand for 2 minutes.
- ☐ 20 Transfer 30 µl from the BIND1 MIDI plate to the PL PCR plate.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the PL PCR plate and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 30 days.

Quantify Libraries (Optional) (Acculear)

- ☐ 1 Combine 6 µl AccuClear DNA standard with 44 ul RSB to dilute DNA standard to 3 ng/µl.
- ☐ 2 Use RSB as blank.
- ☐ 3 Run the diluted AccuClear DNA standard and the blank in triplicate.
- ☐ 4 Run libraries in single replicates.
- ☐ 5 Determine the average relative fluorescence unit (RFU) for the AccuClear DNA standard and the blank.
- ☐ 6 Calculate the following values.
  - Average Standard RFU - Average Blank RFU = Normalized Standard RFU
  - Library RFU - Average Blank RFU = Normalized RFU for each library

Fluorescence Measurement	Recommendation
≤ Average Blank RFU	Repeat library preparation and enrichment if purified DNA sample meets quantity and quality specifications.
> Average Blank RFU (and) < Normalized Standard RFU	Proceed to *Normalize Libraries*. Note: Using libraries with RFU below the Normalized Standard RFU might not yield adequate sequencing results to confidently call variants that can be present in the sample.
≥ Normalized Standard RFU	Proceed to *Normalize Libraries*.

Normalize Libraries

- ☐ 1 If the PL PCR plate was stored at -25°C to -15°C, prepare it as follows.
  - ☐ a Thaw at room temperature.
  - ☐ b Centrifuge at 280 × g for 1 minute.
  - ☐ c Pipette to mix and centrifuge.
- ☐ 2 Label a new 96-well MIDI plate BBN (Bead-Based Normalization).
- ☐ 3 Label a new 96-well PCR plate NL (Normalized Libraries).
- ☐ 4 Vortex LNB1 for 1 minute, and then pipette 10 times to mix.
- ☐ 5 Invert LNB1 after mixing to confirm bead pellet resuspension.
- ☐ 6 Combine the following reagents in a new microcentrifuge tube to create LNA1+LNB1 Master Mix:

Master Mix Component	3 Libraries (µl)	24 Libraries (µl)
LNA1	132	1056
LNB1	24	192

- ☐ 7 Vortex to mix.
- ☐ 8 Combine the following reagents in a new microcentrifuge tube to create a fresh EE2+HP3 Elution Mix:

Elution Mix Component	3 Libraries (µl)	24 Libraries (µl)
EE2	114	912
HP3	6	48

- ☐ 9 Vortex to mix.
- ☐ 10 Vortex LNA1+LNB1 Master Mix.
- ☐ 11 Add 45 µl LNA1+LNB1 Master Mix to the BBN MIDI plate.
- ☐ 12 Add 20 µl from the PL PCR plate to the BBN MIDI plate.
- ☐ 13 Shake the plate at 1800 rpm for 10 minutes.
- ☐ 14 Place the BBN MIDI plate on a magnetic stand for 2 minutes.
- ☐ 15 Remove and discard all supernatant.
- ☐ 16 Remove the BBN MIDI plate from the magnetic stand.
- ☐ 17 Add 45 µl LNW1.
- ☐ 18 Shake at 1800 rpm for 2 minutes.
- ☐ 19 Place on a magnetic stand for 2 minutes.
- ☐ 20 Remove and discard all supernatant.
- ☐ 21 Repeat steps 16-20 to wash a **second** time.
- ☐ 22 Use a P20 pipette with fine tips to remove any residual supernatant.
- ☐ 23 Remove the BBN MIDI plate from the magnetic stand.
- ☐ 24 Add 32 µl EE2+HP3 Elution Mix
- ☐ 25 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 26 Place BBN MIDI plate on a magnetic stand for 2 minutes.
- ☐ 27 Transfer 30 µl from the BBN MIDI plate to the NL PCR plate.
- ☐ 28 Add 30 µl LNS1 to the NL PCR plate.
- ☐ 29 Pipette up and down to mix.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the NL PCR plate and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 30 days.

Pool Libraries and Dilute to the Loading Concentration

- ☐ 1 Pool, denature, and dilute libraries to the loading concentration.

Acronyms

Acronym	Definition
ALS	Amplified Library Samples
BBN	Bead-Based Normalization
CAP1	Capture 1
CAP2	Capture 2
cfDNA	Cell-free DNA
ctDNA	Circulating Tumor DNA
ELU1	Elution 1
ELU2	Elution 2
HYB1	Hybridization 1
HYB2	Hybridization 2
LP	Library Preparation
LS	Library Samples
NL	Normalized Libraries
PL	Purified Libraries