Denature and Anneal RNA

☐ 1 Combine the following volumes :

Master Mix Component	3 Samples (µl)	24 Samples (µl)
FSM	27	216
RVT	3	24

☐ 2 Pipette to mix.
☐ 3 Place the FSM+RVT Master Mix on ice.
☐ 4 Add 8.5 µl of each purified RNA sample to the CF PCR plate.
☐ 5 Add 8.5 µl EPH3.
☐ 6 Shake the plate at 1200 rpm for 1 minute.
☐ 7 Centrifuge at 280 × g for 1 minute.
☐ 8 Place on the thermal cycler and run the LQ-RNA or HQ-RNA program.

Synthesize First Strand cDNA

☐ 1 Remove the CF PCR plate from the thermal cycler.
☐ 2 Pipette FSM+RVT Master Mix to mix.
☐ 3 Add 8 µl FSM+RVT Master Mix .
☐ 4 Pipette 5 times to mix.
☐ 5 Shake the plate at 1200 rpm for 1 minute.
☐ 6 Centrifuge at 280 × g for 1 minute.
☐ 7 Place on the thermal cycler and run the 1stSS program.

Synthesize Second Strand cDNA

☐ 1 Remove the CF PCR plate from the thermal cycler.
☐ 2 Add 25 µl SSM.
☐ 3 Shake the plate at 1200 rpm for 1 minute.
☐ 4 Place on the thermal cycler and run the 2ndSS program.

Clean Up cDNA

- ☐ 1 Remove the CF PCR plate from the thermal cycler.
- ☐ 2 Vortex SPB for 1 minute to resuspend the beads.
- ☐ 3 Add 90 µl SPB to the BIND1 MIDI plate.
- ☐ 4 Transfer 50 µl of each sample from the CF PCR plate to the BIND1 MIDI plate.
- ☐ 5 Shake at 1800 rpm for 2 minutes.
- ☐ 6 Incubate at room temperature for 5 minutes.
- ☐ 7 Place the BIND1 MIDI plate on a magnetic stand for 5 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 the BIND1 MIDI plate from the magnetic stand.
- ☐ 13 Add 22 µl RSB.
- ☐ 14 Shake at 1800 rpm for 2 minutes.
- ☐ 15 Incubate at room temperature for 2 minutes.
- ☐ 16 Place on a magnetic stand for 2 minutes.
- ☐ 17 Transfer 20 µl eluate from the BIND1 MIDI plate to the PCF plate.
- ☐ 18 Add 30 µl RSB to the PCF plate, and then pipette at least 10 times to mix.
- ☐ 19 Select one of the following options based on sample type:
  - **DNA and RNA samples**— Proceed to *Fragment gDNA* on page 2 or follow instructions at the safe stopping point.
  - **RNA samples only**— If you are processing sample from RNA only, and not stopping at the safe stopping point, proceed to *Perform End Repair and A-Tailing* on page 3.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the PCF PCR plate, and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 7 days.

Fragment gDNA

- ☐ 1 Add 12 µl of each gDNA sample into a Covaris 8 microTUBE Strip.
- ☐ 2 Add 40 µl TEB.
- ☐ 3 Pipette to mix.
- ☐ 4 Seal the microTUBE Strip.
- ☐ 5 Centrifuge briefly.
- ☐ 6 Fragment the gDNA using the following settings.

Setting	E220 *evolution*	LE220	ME220
Peak Incident Power	175 watts	450 watts	50 watts
Duty Factor	10%	30%	30%
Cycles per Burst	200	200	1000
Treatment Time	280 seconds	250 seconds	10 seconds
Temperature	7°C	7°C	12°C
Intensifier	yes	N/A	N/A
Other	Intensifier	N/A	Wave guide
Pulse repeats	N/A	N/A	20
Average power	N/A	N/A	15 watts

- ☐ 7 Centrifuge tube strip briefly to collect droplets.
- ☐ 8 Transfer 50 µl of each sheared gDNA sample to the LP plate (or PCF plate if you are processing cDNA simultaneously).

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the LP or PCF plate and briefly centrifuge at 280 × g. Store at -25°C to -15°C for up to 7 days.

Perform End Repair and A-Tailing

- ☐ 1 Combine the following volumes in a microcentrifuge tube to prepare ERA1 Master Mix:

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

- ☐ 2 Pipette 10 times to mix, and then place ERA1 Master Mix on ice.
- ☐ 3 Add 10 µl ERA1 Master Mix to each sample in the LP2 MIDI plate.
- ☐ 4 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 5 Incubate at 30°C for 30 minutes.
- ☐ 6 Immediately transfer to another incubator at 72°C and incubate for 20 minutes.
- ☐ 7 Place the plate on ice for 5 minutes.

Ligate Adapters

- ☐ 1 Add 60 µl ALB1.
- ☐ 2 Add 5 µl LIG3.
- ☐ 3 Add the appropriate adapters.
  - For DNA libraries only, add 10 µl UMI1.
  - For RNA libraries only, add 10 µl SUA1.
- ☐ 4 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 5 Incubate at room temperature for 30 minutes.
- ☐ 6 Add 5 µl STL.
- ☐ 7 Shake the plate at 1800 rpm for 2 minutes.

Clean Up Ligation

- ☐ 1 Vortex SPB for 1 minute to resuspend the beads.
- ☐ 2 Add 112 µl SPB to the LP2 MIDI plate.
- ☐ 3 Shake at 1800 rpm for 2 minutes.
- ☐ 4 Incubate at room temperature for 5 minutes.
- ☐ 5 Place the LP2 MIDI plate on the magnetic stand for 10 minutes.
- ☐ 6 Remove and discard all supernatant.
- ☐ 7 Add 200 µl EtOH, and then remove EtOH after 30 seconds.
- ☐ 8 Repeat step 7 to wash a **second** time.
- ☐ 9 Use a P20 pipette with fine tips to remove residual supernatant.
- ☐ 10 Remove from the magnetic stand.
- ☐ 11 Add 27.5 µl RSB.
- ☐ 12 Shake at 1800 rpm for 2 minutes.
- ☐ 13 Incubate at room temperature for 2 minutes.
- ☐ 14 Place on a magnetic stand for 2 minutes.
- ☐ 15 Transfer 25 µl of each eluate from the LP2 MIDI plate to the LS PCR plate.

Index PCR

- ☐ 1 Add 5 µl indexing primer (UPxx) to the LS PCR plate. Apply a new tube cap.
- ☐ 2 Add 20 µl EPM.
- ☐ 3 Shake the plate at 1200rpm for 1 minute.
- ☐ 4 Briefly centrifuge at 280 × g.
- ☐ 5 Place on the thermal cycler and run the I-PCR program.
- ☐ 6 Relabel the plate ALS.
- ☐ 7 Centrifuge briefly.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the ALS plate and store at -25°C to -15°C for up to 30 days.

Set Up First Hybridization

- ☐ 1 Transfer 20 µl of each library to the HYB1 PCR plate.
- ☐ 2 Add 15 µl TCB1.
- ☐ 3 Add 10 µl TCA1.
- ☐ 4 Add the appropriate probe.
  - For DNA libraries, add 5 µl OPD2 (yellow cap).
  - For RNA libraries, add 5 µl OPR1 (red cap).
- ☐ 5 Shake the plate at 1200 rpm for 2 minutes.
- ☐ 6 Place on the thermal cycler and run the HYB1 program. Hybridize for 8—24 hours (overnight) at 57°C.

Capture Targets One

- ☐ 1 Remove the HYB1 PCR plate from the thermal cycler.
- ☐ 2 Vortex SMB for 1 minute to resuspend the beads.
- ☐ 3 Add 150 µl SMB to the CAP1 MIDI plate.
- ☐ 4 Transfer 50 µl from the HYB1 PCR plate to the CAP1 MIDI plate.
- ☐ 5 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 6 Incubate in a Hybex incubator at 57°C for 25 minutes.
- ☐ 7 Place on a magnetic stand for 2 minutes.
- ☐ 8 Remove and discard all supernatant.
- ☐ 9 Remove the CAP1 MIDI plate from the magnetic stand.
- ☐ 10 Add 200 µl EEW.
- ☐ 11 Pipette to mix 10 times.
- ☐ 12 Shake at 1800 rpm for 4 minutes.
- ☐ 13 Incubate in a Hybex incubator at 57°C for 5 minutes.
- ☐ 14 Place on a magnetic stand for 2 minutes.
- ☐ 15 Remove and discard all supernatant.
- ☐ 16 Repeat steps 9–15 to wash a **second** time.
- ☐ 17 Repeat steps 9–15 to wash a **third** time.
- ☐ 18 Use a P20 pipette with fine tips to remove any residual supernatant.
- ☐ 19 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

- ☐ 20 Vortex briefly.
- ☐ 21 Remove the CAP1 MIDI plate from the magnetic stand.
- ☐ 22 17 µl EE2+HP3 Elution Mix.
- ☐ 23 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 24 Place on a magnetic stand for 2 minutes.
- ☐ 25 Transfer 15 µl eluate from the CAP1 MIDI plate to the ELU1 PCR plate.
- ☐ 26 Add 5 µl ET2 to the ELU1 PCR plate.
- ☐ 27 Shake the plate at 1200 rpm for 2 minutes.

Set Up Second Hybridization

- ☐ 1 Add 15 µl TCB1 to the ELU1 PCR plate.
- ☐ 2 Add 10 µl TCA1.
- ☐ 3 Add the appropriate probe.
  - For DNA libraries, add 5 µl OPD2 (yellow cap).
  - For RNA libraries, add 5 µl OPR1 (red cap).
- ☐ 4 Shake the plate at 1200 rpm for 2 minutes.
- ☐ 5 Place on the thermal cycler and run the HYB2 program. Hybridize at 57°C for 1.5–4 hours.

Capture Targets Two

- ☐ 1 Remove the ELU1 PCR plate from the thermal cycler.
- ☐ 2 Vortex SMB for 1 minute to resuspend the beads.
- ☐ 3 Add 150 µl SMB to the CAP2 MIDI plate.
- ☐ 4 Transfer 50 µl from the ELU1 PCR plate to the CAP2 MIDI plate.
- ☐ 5 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 6 Incubate in a Hybex incubator at 57°C for 25 minutes.
- ☐ 7 Place on a magnetic stand for 2 minutes.
- ☐ 8 Remove and discard all supernatant.
- ☐ 9 Remove the CAP2 MIDI plate from the magnetic stand.
- ☐ 10 Add 200 µl RSB.
- ☐ 11 Shake the plate at 1800 rpm for 4 minutes.
- ☐ 12 Place on a magnetic stand for 2 minutes.
- ☐ 13 Remove and discard all supernatant.
- ☐ 14 Use a P20 pipette with fine tips to remove any residual supernatant.
- ☐ 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	627
HP3	5	33

- ☐ 16 Vortex to mix.
- ☐ 17 Remove the CAP2 MIDI plate from the magnetic stand.
- ☐ 18 Add 22 µl EE2+HP3 Elution Mix.
- ☐ 19 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 20 Place on a magnetic stand for 2 minutes.
- ☐ 21 Transfer 20 µl eluate from the CAP2 MIDI plate to the ELU2 PCR plate.
- ☐ 22 Add 5 µl ET2 to the ELU2 PCR plate.
- ☐ 23 Shake the plate at 1200 rpm for 2 minutes.
- ☐ 24 Centrifuge briefly.

SAFE STOPPING POINT

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

Amplify Enriched Library

- ☐ 1 Add 5 µl PPC3 to the ELU2 PCR plate.
- ☐ 2 Add 20 µl EPM.
- ☐ 3 Shake the plate at 1200 rpm for 2 minutes.
- ☐ 4 Briefly centrifuge at 280 × g.
- ☐ 5 Place on the preprogrammed thermal cycler and run the EL-PCR program.

Clean Up Amplified Enriched Library

- ☐ 1 Remove the ELU2 PCR plate from the thermal cycler.
- ☐ 2 Vortex SPB for 1 minute to resuspend the beads.
- ☐ 3 Add 110 µl SPB to the BIND2 MIDI plate.
- ☐ 4 Transfer 50 µl from the ELU2 PCR plate to the BIND2 MIDI plate.
- ☐ 5 Shake at 1800 rpm for 2 minutes.
- ☐ 6 Incubate at room temperature for 5 minutes.
- ☐ 7 Place the BIND2 MIDI plate on magnetic stand for 5 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 the BIND2 MIDI plate from the magnetic stand.
- ☐ 13 Add 32 µl RSB.
- ☐ 14 Shake at 1800 rpm for 2 minutes.
- ☐ 15 Incubate at room temperature for 2 minutes.
- ☐ 16 Place on a magnetic stand for 2 minutes.
- ☐ 17 Transfer 30 µl from the BIND2 MIDI plate to the PL PCR plate.

SAFE STOPPING POINT

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

Normalize Libraries

- ☐ 1 Pulse vortex LNB1 tube for 1 minute at maximum speed. Invert tube to make sure all beads are resuspended.
- ☐ 2 Set a P1000 to 800 µl and pipette LNB1 tube 10 times to mix.
- ☐ 3 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

- ☐ 4 Vortex to mix.
- ☐ 5 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

- ☐ 6 Vortex to mix.
- ☐ 7 Vortex LNA1+LNB1 Master Mix.
- ☐ 8 Add 45 µl LNA1+LNB1 Master Mix to the BBN MIDI plate.
- ☐ 9 Add 20 µl from the PL PCR plate to the BBN MIDI plate.
- ☐ 10 Shake at 1800 rpm for 30 minutes.
- ☐ 11 Place the BBN MIDI plate on a magnetic stand for 2 minutes.
- ☐ 12 Remove and discard all supernatant.
- ☐ 13 Remove the BBN MIDI plate from the magnetic stand.
- ☐ 14 Add 45 µl LNW1.
- ☐ 15 Shake at 1800 rpm for 5 minutes.
- ☐ 16 Place on a magnetic stand for 2 minutes.
- ☐ 17 Remove and discard all supernatant.
- ☐ 18 Repeat steps 13–17 to wash a **second** time.
- ☐ 19 Use a P20 pipette with fine tips to remove any residual supernatant.
- ☐ 20 Remove the BBN MIDI plate from the magnetic stand.
- ☐ 21 Add 32 µl EE2+HP3 Elution Mix
- ☐ 22 Shake at 1800 rpm for 2 minutes.
- ☐ 23 Place BBN MIDI plate on a magnetic stand for 2 minutes.
- ☐ 24 Transfer 30 µl from the BBN MIDI plate to the NL PCR plate.
- ☐ 25 Add 30 µl LNS1 to the NL PCR plate.
- ☐ 26 Pipette up and down to mix.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the NL 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
1stSS	1st Strand Synthesis
2ndSS	2nd Strand Synthesis
ALS	Amplified Library Samples
BBN	Bead Based Normalization
CAP1	Capture 1
CAP2	Capture 2
cDNA	Complementary DNA
CF	cDNA Fragments
ELU1	Elution 1
ELU2	Elution 2
gDNA	Genomic DNA
HQ-RNA	High-quality RNA
HYB1	Hybridization 1
HYB2	Hybridization 2
LP	Library Preparation
LP2	Library Preparation 2
LQ-RNA	Low-quality RNA
LS	Library Samples
NL	Normalized Libraries
PCF	Purified cDNA Fragments
PL	Purified Libraries