Denature and Anneal RNA

☐1 Combine the following reagents in a microcentrifuge tube to create FSM+RVT master mix.

Master Mix Component	Per 3 Samples	Per 24 Samples
FSM	27 µl	216 µl
RVT	3 µl	24 µl

☐2 Pipette to mix.
☐3 Place the FSM+RVT master mix on ice until *Synthesize First Strand cDNA* on page 1.
☐4 Add 8.5 µl of each purified RNA sample (4.7 ng/µl to 10 ng/µl) to the CF plate.
☐5 Add 8.5 µl EPH3.
☐6 Shake the plate at 1200 rpm for 1 minute.
☐7 Place on the thermal cycler and run the LQ-RNA or HQ-RNA program.
☐8 When the thermal cycler reaches 4°C, proceed immediately to *Synthesize First Strand cDNA* on page 1.

Synthesize First Strand cDNA

☐1 Remove the CF plate from the thermal cycler.
☐2 Pipette to mix FSM+RVT master mix before use.
☐3 Add 8 µl FSM+RVT master mix .
☐4 Shake the plate at 1200 rpm for 1 minute.
☐5 Place on a thermal cycler and run the 1stSS program.
☐6 When the thermal cycler reaches 4°C, proceed immediately to *Synthesize Second Strand cDNA* on page 1.

Synthesize Second Strand cDNA

☐1 Remove the CF plate from the thermal cycler.
☐2 Add 25 µl SSM.
☐3 Shake the plate at 1200 rpm for 1 minute.
☐4 Place on a thermal cycler and run the 2ndSS program.
☐5 When the thermal cycler reaches 4°C, proceed to *Clean Up cDNA* on page 2.

Clean Up cDNA

- ☐ 1 Remove the CF plate from the thermal cycler.
- ☐ 2 Add 90 µl SPB to the BIND1 midi plate.
- ☐ 3 Transfer 50 µl of each sample from the CF plate to the BIND1 midi plate.
- ☐ 4 Shake at 1800 rpm for 2 minutes.
- ☐ 5 Incubate at room temperature for 5 minutes.
- ☐ 6 Place the BIND1 midi plate on a magnetic stand for 5 minutes.
- ☐ 7 Remove and discard all supernatant.
- ☐ 8 Add 200 µl EtOH, and then remove EtOH after 30 seconds.
- ☐ 9 Repeat step 8 to wash a second time.
- ☐ 10 Use a P20 pipette with fine tips to remove residual supernatant.
- ☐ 11 Remove the BIND1 midi plate from the magnetic stand.
- ☐ 12 Add 22 µl RSB.
- ☐ 13 Shake at 1500 rpm for 2 minutes.
- ☐ 14 Incubate at room temperature for 2 minutes.
- ☐ 15 Place on a magnetic stand for 2 minutes.
- ☐ 16 Transfer 20 µl of eluate from the BIND1 midi plate to the PCF plate.
- ☐ 17 Add 30 µl RSB to the PCF plate, and then pipette to mix (a minimum of 10 times).

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the 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
Peak Incident Power	175 watts	450 watts
Duty Factor	10%	30%
Cycles per Burst	200	200
Treatment Time	280 seconds	250 seconds
Temperature	7°C	7°C
Intensifier	yes	N/A

- ☐ 7 Transfer 50 µl of each sheared gDNA sample to the LP plate (or PCF plate if you are processing cDNA simultaneously).
  - A P20 pipette with fine tips can be used when transferring sheared gDNA sample to the LP plate (pipette 20 µl + 20 µl + 10 µl).
- ☐ 8 [Optional] If the PCF plate is a midi plate and you plan to store it after this step, transfer 50 µl of cDNA and 50 µl of sheared gDNA sample to the corresponding wells of a new 96-well PCR plate.
  - A P20 pipette with fine tips can be used when transferring sheared gDNA sample to the LP plate (pipette 20 µl + 20 µl + 10 µl).
  - Label the plate LP.

SAFE STOPPING POINT

If you are stopping, apply Microseal 'B' to the PCR 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 reagents in a microcentrifuge tube to create ERA1 master mix.

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

- ☐ 2 Pipette to mix (a minimum of 10 times) and place ERA1 master mix on ice.
- ☐ 3 Add 10 µl ERA1 master mix to each sample in the LP2 plate.
- ☐ 4 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 5 Incubate in a Hybex incubator at 30°C for 30 minutes.
- ☐ 6 Immediately transfer to another Hybex 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 Vortex SUA1.
- ☐ 4 Add 10 µl SUA1.
- ☐ 5 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 6 Incubate at room temperature for 30 minutes.
- ☐ 7 Add 5 µl STL to each well.
- ☐ 8 Shake the plate at 1800 rpm for 2 minutes.

Clean Up Ligation

- ☐ 1 Add 112 µl SPB to the LP2 plate.
- ☐ 2 Shake at 1800 rpm for 2 minutes.
- ☐ 3 Incubate at room temperature for 5 minutes.
- ☐ 4 Place the LP2 plate on the magnetic stand for 10 minutes.
- ☐ 5 Remove and discard all supernatant.
- ☐ 6 Add 200 µl EtOH, and then remove EtOH after 30 seconds.
- ☐ 7 Repeat step 6 to wash a second time.
- ☐ 8 Use a P20 pipette with fine tips to remove residual supernatant.
- ☐ 9 Remove from the magnetic stand.
- ☐ 10 Add 27.5 µl RSB.
- ☐ 11 Shake at 1500 rpm for 2 minutes.
- ☐ 12 Incubate at room temperature for 2 minutes.
- ☐ 13 Place on a magnetic stand for 2 minutes.
- ☐ 14 Transfer 25 µl of each eluate from the LP2 plate to the LS plate.

Index PCR

- ☐ 1 Add 5 µl of indexing primer mix (UPXX or CPXX) to the LS plate.
- ☐ 2 Add 20 µl EPM.
- ☐ 3 Shake the plate at 1500 rpm for 1 minute.
- ☐ 4 Briefly centrifuge at 280 × g.
- ☐ 5 Place on the preprogrammed thermal cycler and run the I-PCR program.
- ☐ 6 After the I-PCR program completes, relabel the plate ALS.
- ☐ 7 Centrifuge briefly.

SAFE STOPPING POINT

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

Perform First Hybridization

- ☐ 1 Transfer 20 µl of each RNA and/or DNA library to the HYB1 plate.
- ☐ 2 Add 15 µl TCB1.
- ☐ 3 Add 10 µl TCA1.
- ☐ 4 Add the appropriate probe:
  - For RNA libraries, add 5 µl of OPR1 (red cap).
  - For DNA libraries, add 5 µl of OPD1 (blue cap).
- ☐ 5 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 6 Place on the thermal cycler and run the HYB1 program. Hybridize at 57°C overnight (minimum of 8 hours to a maximum of 24 hours).

Perform First Capture

- ☐ 1 Remove the HYB1 plate from the thermal cycler.
- ☐ 2 Add 150 µl SMB to the CAP1 plate.
- ☐ 3 Transfer 50 µl from the HYB1 plate to the CAP1 plate.
- ☐ 4 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 5 Incubate in a Hybex incubator at 57°C for 25 minutes.
- ☐ 6 Place on a magnetic stand for 2 minutes.
- ☐ 7 Remove and discard all supernatant.
- ☐ 8 Remove the CAP1 plate from the magnetic stand.
- ☐ 9 Add 200 µl EEW2.
- ☐ 10 Pipette to mix 10 times.
- ☐ 11 Shake at 1800 rpm for 4 minutes.
- ☐ 12 Incubate in a Hybex incubator at 57°C for 5 minutes.
- ☐ 13 Place on a magnetic stand for 2 minutes.
- ☐ 14 Remove and discard all supernatant.
- ☐ 15 Repeat steps 8–14 to wash a second time.
- ☐ 16 Repeat steps 8–14 to wash a third time.
- ☐ 17 Use a P20 pipette with fine tips to remove any residual supernatant.
- ☐ 18 Combine the following reagents in a microcentrifuge tube to create EE2+HP3 Elution Mix.

Elution Mix Component	Per 3 Samples	Per 24 Samples
EE2	95 µl	684 µl
HP3	5 µl	36 µl

- ☐ 19 Vortex briefly.
- ☐ 20 Remove the CAP1 plate from the magnetic stand.
- ☐ 21 Add 17 µl EE2+HP3 Elution Mix.

- ☐ 22 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 23 Place on a magnetic stand for 2 minutes.
- ☐ 24 Transfer 15 µl of eluate from the CAP1 plate into the ELU1 plate.
- ☐ 25 Add 5 µl ET2 to the ELU1 plate.
- ☐ 26 Shake the plate at 1800 rpm for 2 minutes.

Perform Second Hybridization

- ☐ 1 Add 15 µl TCB1 to the ELU1 plate.
- ☐ 2 Add 10 µl TCA1.
- ☐ 3 Add the appropriate probe:
  - For RNA libraries, add 5 µl OPR1 (red cap).
  - For DNA libraries, add 5 µl OPD1 (blue cap).
- ☐ 4 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 5 Place on the thermal cycler and run the HYB2 program. Hybridize at 57°C for a minimum of 1.5 hours to a maximum of 4 hours.

Perform Second Capture

- ☐ 1 Remove the ELU1 plate from the thermal cycler.
- ☐ 2 Add 150 µl SMB to the CAP2 plate.
- ☐ 3 Transfer 50 µl from the ELU1 plate to the CAP2 plate.
- ☐ 4 Shake the plate at 1800 rpm for 2 minutes.
- ☐ 5 Incubate in a Hybex incubator at 57°C for 25 minutes.
- ☐ 6 Place on a magnetic stand for 2 minutes.
- ☐ 7 Remove and discard all supernatant.
- ☐ 8 Remove the CAP2 plate from the magnetic stand.
- ☐ 9 Add 200 µl of RSB.
- ☐ 10 Shake the plate at 1800 rpm for 4 minutes.
- ☐ 11 Place on a magnetic stand for 2 minutes.
- ☐ 12 Remove and discard all supernatant.
- ☐ 13 Use a P20 pipette with fine tips to remove any residual supernatant.
- ☐ 14 Combine the following reagents in a microcentrifuge tube to create a fresh EE2+HP3 Elution Mix.

Elution Mix Component	Per 3 Samples	Per 24 Samples
EE2	95 µl	684 µl
HP3	5 µl	36 µl

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

☐ 22 Shake the plate at 1800 rpm for 2 minutes.

SAFE STOPPING POINT

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

Amplify Enriched Library

☐ 1 Add 5 µl PPC3 to the ELU2 plate.
☐ 2 Add 20 µl EPM.
☐ 3 Shake the plate at 1500 rpm for 2 minutes.
☐ 4 Briefly centrifuge at 280 × g.
☐ 5 Place on a thermal cycler and run the EL-PCR program.

Clean Up Amplified Enriched Library

☐ 1 Remove the ELU2 plate from the thermal cycler.
☐ 2 Add 110 µl SPB to the BIND2 plate.
☐ 3 Transfer 50 µl from the ELU2 plate to the BIND2 plate.
☐ 4 Shake at 1800 rpm for 2 minutes.
☐ 5 Incubate at room temperature for 5 minutes.
☐ 6 Place the BIND2 plate on magnetic stand for 5 minutes.
☐ 7 Remove and discard all supernatant.
☐ 8 Add 200 µl EtOH, and then remove EtOH after 30 seconds.
☐ 9 Repeat step 8 to wash a second time.
☐ 10 Use a P20 pipette with fine tips to remove residual supernatant.
☐ 11 Remove the BIND2 plate from the magnetic stand.
☐ 12 Add 32 µl RSB.
☐ 13 Shake at 1800 rpm for 2 minutes.
☐ 14 Incubate at room temperature for 2 minutes.
☐ 15 Place on a magnetic stand for 2 minutes.
☐ 16 Transfer 30 µl from the BIND2 plate to the PL plate.

SAFE STOPPING POINT

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

Normalize Libraries

☐1 Combine the following reagents in a new microcentrifuge tube to create LNA1+LNB1 master mix.

Master Mix Component	Per 3 Samples	Per 24 Samples
LNA1	132 µl	1056 µl
LNB1	24 µl	192 µl

☐2 Vortex to mix.

☐3 Combine the following reagents in a new microcentrifuge tube to create a fresh 0.1 N NaOH solution.

Solution Component	Per 3 Samples	Per 24 Samples
PCR-grade water	114 µl	912 µl
HP3	6 µl	48 µl

☐4 Vortex to mix.

☐5 Add 45 µl LNA1+LNB1 master mix to the BIND3 plate.

☐6 Add 20 µl from the PL plate to the BIND3 plate.

☐7 Shake at 1800 rpm for 10 minutes.

☐8 Place the BIND3 plate on a magnetic stand for 2 minutes.

☐9 Remove and discard all supernatant.

☐10 Remove the BIND3 plate from the magnetic stand.

☐11 Add 45 µl of LNW1.

☐12 Shake at 1800 rpm for 2 minutes.

☐13 Place on a magnetic stand for 2 minutes.

☐14 Remove and discard all supernatant.

☐15 Repeat steps 10–14 to wash a second time.

☐16 Use a P20 pipette with fine tips to remove any residual supernatant.

☐17 Add 32 µl 0.1 N NaOH solution.

☐18 Shake at 1800 rpm for 2 minutes.

☐19 Place BIND3 on a magnetic stand for 2 minutes.

☐20 Transfer 30 µl from the BIND3 plate to the NL plate.

☐21 Add 30 µl LNS2 to the NL plate.

☐22 Pipette up and down to mix.

SAFE STOPPING POINT

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

Pool Libraries

☐1 Transfer 10 µl of RNA library from the NL plate to the PRL tube.

☐2 Transfer 10 µl of DNA library from the NL plate to the PDL tube.

☐3 Vortex to mix.

☐4 Centrifuge briefly.

Dilute Libraries to the Loading Concentration

☐1 Dilute to the final loading concentration.

Acronyms

Acronym	Definition
1stSS	1st Strand Synthesis
2ndSS	2nd Strand Synthesis
ALS	Amplified Library Samples
cDNA	Complementary DNA
CF	cDNA Fragments
ELU1	Elution 1
ELU2	Elution 2
gDNA	Genomic DNA
HQ-RNA	High-quality RNA
HYB1	Hybridization 1
LQ-RNA	Low-quality RNA
LS	Library Samples
LP	Library Preparation
LP2	Library Preparation 2
NL	Normalized Libraries
PCF	Purified cDNA Fragments
PDL	Pooled DNA Libraries
PL	Purified Libraries
PRL	Pooled RNA Libraries