Purify and Fragment mRNA

- ☐ 1 Dilute 25–1000 ng total RNA in nuclease-free ultrapure water to 25 µl.
- ☐ 2 Add 25 µl RPB.
- ☐ 3 Mix using either method:
  - Shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
  - Pipette 10 times.
- ☐ 4 Place on the thermal cycler and run the mRNA_CAP program.
- ☐ 5 Centrifuge at 280 × g for 10 seconds.
- ☐ 6 Place on the magnetic stand and wait 2 minutes.
- ☐ 7 Remove and discard supernatant.
- ☐ 8 Remove from the magnetic stand.
- ☐ 9 Add 100 µl BWB.
- ☐ 10 Mix using either method:
  - Shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
  - Pipette 10 times.
- ☐ 11 Place on the magnetic stand and wait 2 minutes.
- ☐ 12 Remove and discard supernatant.
- ☐ 13 With a 20 µl pipette, remove all residual BWB.
- ☐ 14 Remove from the magnetic stand.
- ☐ 15 Add 25 µl ELB.
- ☐ 16 Mix using either method:
  - Shake at 2200 rpm for 1 minute.
  - Pipette until resuspended.
- ☐ 17 If shaking did not fully resuspend, pipette until resuspended.
- ☐ 18 Centrifuge at 280 × g for 10 seconds.
- ☐ 19 Place on the thermal cycler and run the mRNA_ELT program.
- ☐ 20 Combine the following volumes to prepare Fragmentation Master Mix.
  - Nuclease-free ultrapure water (10.5 µl)
  - EPH3 (10.5 µl)
- ☐ 21 Centrifuge the sealed PCR plate at 280 × g for 10 seconds.
- ☐ 22 Add 25 µl BBB to each well.
- ☐ 23 Mix using either method:
  - Shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
  - Pipette 10 times.
- ☐ 24 Incubate at room temperature for 5 minutes.
- ☐ 25 Place on the magnetic stand and wait 2 minutes.
- ☐ 26 Remove and discard 50 µl supernatant.
- ☐ 27 Remove from the magnetic stand.
- ☐ 28 Add 100 µl BWB.
- ☐ 29 Mix using either method:
  - Shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
  - Pipette 10 times.
- ☐ 30 Place on the magnetic stand and wait 2 minutes.
- ☐ 31 Remove and discard supernatant.
- ☐ 32 Remove residual BWB.
- ☐ 33 Remove from the magnetic stand.
- ☐ 34 Thoroughly pipette Fragmentation Master Mix.
- ☐ 35 Add 19 µl Fragmentation Master Mix.
- ☐ 36 Mix using either method:
  - Shake at 2200 rpm for 1 minute.
  - Pipette until resuspended.
- ☐ 37 If shaking did not fully resuspend, pipette until resuspended.
- ☐ 38 Incubate at room temperature for 2 minutes.
- ☐ 39 Centrifuge at 280 × g for 10 seconds.
- ☐ 40 Place on the thermal cycler and run the DEN94_8 program.
- ☐ 41 Centrifuge at 280 × g for 10 seconds.
- ☐ 42 Place on the magnetic stand and wait 2 minutes.
- ☐ 43 Transfer 17 µl supernatant.
- ☐ 44 Set aside on ice.

Synthesize First Strand cDNA

- ☐ 1 Combine the following volumes to prepare First Strand Synthesis Master Mix.
  - FSA (9 µl)
  - RVT (1 µl)
- ☐ 2 Thoroughly pipette First Strand Synthesis Master Mix.
- ☐ 3 Centrifuge at 280 × g for 10 seconds.
- ☐ 4 Add 8 µl First Strand Synthesis Master Mix.
- ☐ 5 Pipette 10 times.
- ☐ 6 Place on the thermal cycler and run the FSS program.

Synthesize Second Strand cDNA

- ☐ 1 Centrifuge at 280 × g for 10 seconds.
- ☐ 2 Add 25 µl SMM.
- ☐ 3 Pipette 10 times.
- ☐ 4 Place on the thermal cycler and run the SSS program.
- ☐ 5 Centrifuge at 280 × g for 10 seconds.
- ☐ 6 Add 90 µl AMPure XP.
- ☐ 7 Mix using either method:
  - Shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
  - Pipette until resuspended.
- ☐ 8 Incubate at room temperature for 5 minutes.
- ☐ 9 Place on the magnetic stand and wait 5 minutes.
- ☐ 10 Remove and discard 130 µl supernatant.
- ☐ 11 Wash beads as follows.
  - ☐ a Add 175 µl fresh 80% EtOH.
  - ☐ b Wait 30 seconds.
  - ☐ c Remove and discard supernatant.
- ☐ 12 Repeat wash a **second** time.
- ☐ 13 Remove residual EtOH.
- ☐ 14 Air-dry for 2 minutes.
- ☐ 15 Remove from the magnetic stand.
- ☐ 16 Add 19.5 µl RSB.
- ☐ 17 Mix using either method:
  - Shake at 2200 rpm for 1 minute.
  - Pipette until resuspended.
- ☐ 18 If shaking did not fully resuspend, pipette until resuspended.
- ☐ 19 Incubate at room temperature for 2 minutes.
- ☐ 20 Centrifuge at 280 × g for 10 seconds.
- ☐ 21 Place on the magnetic stand and wait 2 minutes.
- ☐ 22 Transfer 17.5 µl supernatant.

SAFE STOPPING POINT

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

Adenylate 3′ Ends

- ☐ 1 Add 12.5 µl ATL4.
- ☐ 2 Using a 200 µl pipette, pipette 10 times.
- ☐ 3 Place on the thermal cycler and run the ATAIL program.

Ligate Anchors

- ☐ 1 Centrifuge at 280 × g for 10 seconds.
- ☐ 2 Add the following volumes *in the order listed*.

Reagent	Volume for Input ≤ 100 ng (µl)	Volume for Input > 100 ng (µl)
RSB	2.5	0
RNA Index Anchors	2.5	5
LIGX	2.5	2.5

- ☐ 3 Pipette 10 times, and then seal.
- ☐ 4 Place on the thermal cycler and run the LIG program.
- ☐ 5 Centrifuge at 280 × g for 10 seconds.
- ☐ 6 Add 5 µl STL.
- ☐ 7 Pipette 15 times to mix.

Clean Up Fragments

- ☐ 1 Add 34 µl AMPure XP.
- ☐ 2 Mix using either method:
  - Shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
  - Pipette until resuspended.
- ☐ 3 Incubate at room temperature for 5 minutes.
- ☐ 4 Place on the magnetic stand and wait 5 minutes.
- ☐ 5 Remove and discard 67 µl supernatant.
- ☐ 6 Wash beads as follows.
  - ☐ a Add 175 µl fresh 80% EtOH.
  - ☐ b Wait 30 seconds.
  - ☐ c Remove and discard supernatant.
- ☐ 7 Wash beads a **second** time.
- ☐ 8 Remove residual EtOH.
- ☐ 9 Air-dry for 2 minutes.
- ☐ 10 Remove from the magnetic stand.
- ☐ 11 Add 22 µl RSB.
- ☐ 12 Mix using either method:
  - Shake at 2200 rpm for 1 minute.
  - Pipette until resuspended.
- ☐ 13 If shaking did not resuspend, pipette until resuspended.
- ☐ 14 Incubate at room temperature for 2 minutes.
- ☐ 15 Centrifuge at 280 × g for 10 seconds.
- ☐ 16 Place on the magnetic stand and wait 2 minutes.
- ☐ 17 Transfer 20 µl supernatant

SAFE STOPPING POINT

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

Amplify Library

- ☐ 1 Pierce the index adapter plate wells.
- ☐ 2 Add the following volumes *in the order listed*.
  - UDP0XXX (10 µl)
  - EPM (20 µl)
- ☐ 3 Pipette 10 times.
- ☐ 4 Place on the thermal cycler and run the PCR program.

SAFE STOPPING POINT

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

Clean Up Library

- ☐ 1 Centrifuge at 280 × g for 10 seconds.
- ☐ 2 Add 50 µl AMPure XP.
- ☐ 3 Mix using either method:
  - Shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
  - Pipette until resuspended.
- ☐ 4 Incubate at room temperature for 5 minutes.
- ☐ 5 Place on the magnetic stand and wait 5 minutes.
- ☐ 6 Remove and discard 90 µl supernatant.
- ☐ 7 Wash beads as follows.
  - ☐ a Add 175 µl fresh 80% EtOH.
  - ☐ b Wait 30 seconds.
  - ☐ c Remove and discard supernatant.
- ☐ 8 Wash beads a **second** time.
- ☐ 9 Remove residual EtOH.
- ☐ 10 Air-dry for 2 minutes.
- ☐ 11 Remove from the magnetic stand.
- ☐ 12 Add 17 µl RSB to each well.
- ☐ 13 Mix using either method:
  - Shake at 2200 rpm for 1 minute.
  - Pipette until resuspended.
- ☐ 14 If shaking did not resuspend, pipette until resuspended.
- ☐ 15 Incubate at room temperature for 2 minutes.
- ☐ 16 Centrifuge at 280 × g for 10 seconds.
- ☐ 17 Place on the magnetic stand and wait 2 minutes.
- ☐ 18 Transfer 15 µl supernatant.

SAFE STOPPING POINT

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

Check Library

- ☐ 1 Analyze 1 µl library using the Agilent 2100 Bioanalyzer and DNA 1000 Kit.
- ☐ 2 [Optional] Analyze 2 µl library using the Qubit dsDNA BR Assay Kit.

Dilute Library to the Starting Concentration

☐ 1 Obtain the molarity value:
- **Bioanalyzer quantification only**—Use the molarity value obtained for the library.
- **Bioanalyzer and Qubit quantification**—Calculate molarity value using the average size and concentration.

☐ 2 Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system.

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
NextSeq 550 and NextSeq 500	1	1.1–1.4
NovaSeq 6000	0.5	100

☐ 3 Dilute each library to the starting concentration. Combine 10 µl each diluted library in a tube.

☐ 4 Follow denature and dilute instructions to dilute libraries.

Acronyms

Acronym	Definition
ATL4	A-Tailing Mix
BBB	Bead Binding Buffer
BWB	Bead Washing Buffer
cDNA	Complementary DNA
ELB	Elution Buffer
EPH3	Elute, Prime, Fragment High Concentration Mix
EPM	Enhanced PCR Mix
EtOH	Ethanol
FSA	First Strand Synthesis Act D Mix
LIGX	Ligation Mix
mRNA	Messenger RNA
RPBX	RNA Purification Beads
RSB	Resuspension Buffer
SMM	Second Strand Master Mix
STL	Stop Ligation Buffer