Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus Checklist

Deplete rRNA

- ☐ 1 Using nuclease-free ultrapure water, dilute 1–1000 ng total RNA to 11 µl.
- ☐ 2 Combine the following volumes to prepare Hybridize Probe Master Mix.
  - DB1 (3.6 µl)
  - DP1 (1.2 µl)
- ☐ 3 Thoroughly pipette Hybridize Probe Master Mix.
- ☐ 4 Add 4 µl Hybridize Probe Master Mix.
- ☐ 5 Pipette 10 times.
- ☐ 6 Place on the thermal cycler and run the HYB_DP1 program.
- ☐ 7 Combine the following volumes to prepare rRNA Depletion Master Mix.
  - RDB (4.8 µl)
  - RDE (1.2 µl)
- ☐ 8 Thoroughly pipette rRNA Depletion Master Mix.
- ☐ 9 Centrifuge at 280 × g for 10 seconds.
- ☐ 10 Add 5 µl rRNA Depletion Master Mix.
- ☐ 11 Pipette 10 times.
- ☐ 12 Place on the thermal cycler and run the RNA_DEP program.
- ☐ 13 Combine the following volumes to prepare Probe Removal Master Mix.
  - PRB (7.7 µl)
  - PRE (3.3 µl)
- ☐ 14 Thoroughly pipette Probe Removal Master Mix.
- ☐ 15 Centrifuge at 280 × g for 10 seconds.
- ☐ 16 Add 10 µl Probe Removal Master Mix.
- ☐ 17 Pipette 10 times.
- ☐ 18 Place on the thermal cycler and run the PRB_REM program.
- ☐ 19 Centrifuge at 280 × g for 10 seconds.
- ☐ 20 Add 60 µl RNAClean XP.
- ☐ 21 Mix using either method:
  - Shake at 2000 rpm for 1 minute, and then centrifuge at 280 × g for 10 seconds.
  - Pipette until resuspended.
- ☐ 22 Incubate at room temperature for 5 minutes.
- ☐ 23 Place on the magnetic stand 5 minutes.
- ☐ 24 Remove and discard 80 µl supernatant.
- ☐ 25 Wash beads as follows.
  - ☐ a Add 175 µl fresh 80% EtOH.
  - ☐ b Wait 30 seconds.
  - ☐ c Remove and discard supernatant.
- ☐ 26 Wash beads a **second** time.
- ☐ 27 Remove residual EtOH.
- ☐ 28 Air-dry on the magnetic stand for 2 minutes.
- ☐ 29 Remove from the magnetic stand.
- ☐ 30 Add 10.5 µl ELB.
- ☐ 31 Mix using either method:
  - Centrifuge at 280 × g for 10 seconds, and then shake at 2200 rpm for 1 minute.
  - Pipette until resuspended.
- ☐ 32 If shaking did not resuspend, pipette until resuspended.
- ☐ 33 Incubate at room temperature for 2 minutes.
- ☐ 34 Centrifuge at 280 × g for 10 seconds.
- ☐ 35 Place on the magnetic stand 2 minutes.
- ☐ 36 Transfer 8.5 µl supernatant to a new PCR plate.

Fragment and Denature RNA

- ☐ 1 Add 8.5 µl EPH3.
- ☐ 2 Pipette 10 times.
- ☐ 3 Place on the thermal cycler and run the DEN_RNA program.

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 10 times.
- ☐ 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 Wash beads 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 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.

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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

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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
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.

Trim T-Overhang (Optional)

☐ 1 To trim using the FASTQ Toolkit app on Analysis Software (AS), perform the following actions.
- ☐ a Expand the base trimming settings section.
- ☐ b Enter 1 in the Trim reads at the 5′-end by n positions field.

☐ 2 To trim using a sample sheet, add the following settings to the sample sheet file.
- Read1StartFromCycle,2
- Read2StartFromCycle,2