Illumina RNA Prep with Enrichment, (L) Tagmentation Checklist

Denature RNA

- ☐ 1 Dilute 10–100 ng total RNA in nuclease-free ultrapure water to 8.5 µl.
- ☐ 2 Add 8.5 µl EPH3.
- ☐ 3 Pipette 10 times.
- ☐ 4 Centrifuge at 280 × g for 3 seconds.
- ☐ 5 Place on the thermal cycler and run the DEN_RNA program.
- ☐ 6 Centrifuge at 280 × g for 10 seconds.

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 Add 8 µl First Strand Synthesis Master Mix.
- ☐ 4 Pipette 10 times.
- ☐ 5 Centrifuge at 280 × g for 10 seconds.
- ☐ 6 Place on the thermal cycler and run the FSS program.

Synthesize Second Strand cDNA

- ☐ 1 Centrifuge at 280 × g for 10 seconds.
- ☐ 2 Invert SMM to mix, and then centrifuge briefly.
- ☐ 3 Add 25 µl SMM.
- ☐ 4 Pipette 10 times.
- ☐ 5 Centrifuge at 280 × g for 10 seconds.
- ☐ 6 Place on the thermal cycler and run the SSS program.
- ☐ 7 Centrifuge at 280 × g for 10 seconds.
- ☐ 8 Add 90 µl AMPure XP.
- ☐ 9 Shake at 2200 rpm for 1 minute.
- ☐ 10 Incubate at room temperature for 5 minutes.
- ☐ 11 Centrifuge at 280 × g for 10 seconds, and then unseal.
- ☐ 12 Place on the magnetic stand until liquid is clear.
- ☐ 13 Remove and discard supernatant.
- ☐ 14 Wash beads as follows.
  - ☐ a Add 175 µl fresh 80% EtOH.
  - ☐ b Wait 30 seconds.
  - ☐ c Remove and discard supernatant.
- ☐ 15 Repeat wash a **second** time.
- ☐ 16 Remove residual EtOH.
- ☐ 17 Air-dry for 2 minutes.
- ☐ 18 Remove from the magnetic stand.
- ☐ 19 Add 19.5 µl RSB.
- ☐ 20 Shake at 2700 rpm for 1 minute.
- ☐ 21 Incubate at room temperature for 2 minutes.
- ☐ 22 Centrifuge at 280 × g for 10 seconds, and then unseal.
- ☐ 23 Place on the magnetic stand until liquid is clear.
- ☐ 24 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.

Tagment cDNA

- ☐ 1 Centrifuge at 280 × g for 10 seconds.
- ☐ 2 Combine the following volumes to prepare Tagmentation Master Mix.
  - TB1 (11.5 µl)
  - EBLTL (11.5 µl)
  - Nuclease-free ultrapure water (14.5 µl)
- ☐ 3 Thoroughly vortex the Tagmentation Master Mix.
- ☐ 4 Add 32.5 µl Tagmentation Master Mix.
- ☐ 5 Pipette thoroughly.
- ☐ 6 Place on the thermal cycler and run the TAG program.
- ☐ 7 Centrifuge at 280 × g for 10 seconds.
- ☐ 8 Incubate at room temperature for 2 minutes.
- ☐ 9 Add 10 µl ST2.
- ☐ 10 Shake at 2200 rpm for 1 minute.
- ☐ 11 Incubate at room temperature for 5 minutes.
- ☐ 12 Centrifuge at 280 × g for 10 seconds, and then unseal.
- ☐ 13 Place on the magnetic stand until liquid is clear.
- ☐ 14 Remove and discard supernatant.
- ☐ 15 Wash beads as follows.
  - ☐ a Remove from the magnetic stand.
  - ☐ b Add 100 µl TWB to each well.
  - ☐ c Shake at 2000 rpm for 1 minute.
  - ☐ d Centrifuge at 280 × g for 3 seconds.
  - ☐ e Place on the magnetic stand until liquid is clear.
  - ☐ f Remove and discard supernatant.
- ☐ 16 Wash beads a **second** time.
- ☐ 17 Wash beads a **third** time, ***skipping step f***.
- ☐ 18 Combine the following volumes to prepare PCR Master Mix.
  - EPM (23 µl)
  - Nuclease-free ultrapure water (23 µl)
- ☐ 19 Thoroughly vortex PCR Master Mix.
- ☐ 20 Remove and discard TWB supernatant.
- ☐ 21 Remove residual TWB.
- ☐ 22 Remove from the magnetic stand.
- ☐ 23 Add 40 µl PCR Master Mix.
- ☐ 24 Pierce the index adapter plate wells.
- ☐ 25 Add 10 µl UDP0XXX.
- ☐ 26 Shake at 2000 rpm for 1 minute.
- ☐ 27 Centrifuge at 280 × g for 3 seconds.
- ☐ 28 Place on the thermal cycler and run the TAG_PCR program.

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Illumina RNA Prep with Enrichment, (L) Tagmentation Checklist

Clean Up Library

- ☐ 1 Centrifuge at 280 × g for 10 seconds.
- ☐ 2 Place on the magnetic stand until liquid is clear.
- ☐ 3 Transfer 45 µl supernatant.
- ☐ 4 Add 81 µl AMPure XP.
- ☐ 5 Shake at 2200 rpm for 1 minute.
- ☐ 6 Incubate at room temperature for 5 minutes.
- ☐ 7 Centrifuge at 280 × g for 10 seconds, and then unseal.
- ☐ 8 Place on the magnetic stand until liquid is clear.
- ☐ 9 Remove and discard supernatant.
- ☐ 10 Wash beads as follows.
  - ☐ a Add 175 µl fresh 80% EtOH.
  - ☐ b Wait 30 seconds.
  - ☐ c Remove and discard all supernatant.
- ☐ 11 Wash beads a **second** time.
- ☐ 12 Remove residual EtOH.
- ☐ 13 Air-dry on the magnetic stand for 2 minutes.
- ☐ 14 Remove from the magnetic stand.
- ☐ 15 Add 17 µl RSB.
- ☐ 16 Shake at 2700 rpm for 1 minute.
- ☐ 17 Incubate at room temperature for 2 minutes.
- ☐ 18 Centrifuge at 280 × g for 10 seconds, and then unseal.
- ☐ 19 Place on the magnetic stand until liquid is clear.
- ☐ 20 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 30 days.

Normalize Library

- ☐ 1 Analyze 1 µl library with the Qubit dsDNA BR Assay Kit.
- ☐ 2 **[Optional]** Analyze 1 µl library with the Agilent 2100 Bioanalyzer System and a DNA 1000 Kit.
- ☐ 3 **[Respiratory Virus Panel Libraries]**
  - For one-plex enrichment, transfer 7.5 µl undiluted library to one well.
  - For three-plex enrichment, dilute three 200 ng libraries to 2.5 µl each.
- ☐ 4 **[All Other Libraries]** Dilute libraries in RSB as follows.
  - For one-plex enrichment, dilute one 200 ng library to 7.5 µl.
  - For three-plex enrichment, dilute three 200 ng libraries to 2.5 µl each.
- ☐ 5 **[Diluted Libraries]** In one well, combine the 200 ng libraries:

Number of Libraries	Total Mass (ng)	Total Volume (µl)
1	200	7.5
3	600	7.5

  - If the total volume is > 7.5 µl, concentrate the pooled sample to 7.5 µl.

Hybridize Probes

- ☐ 1 Add the following volumes ***in the order listed***.
  - 200 ng library or 600 ng pool (7.5 µl)
  - NHB2 (12.5 µl)
  - Enrichment oligos (2.5 µl)
  - EHB2 (2.5 µl)
- ☐ 2 Pipette 10 times to mix.
- ☐ 3 Centrifuge at 280 × g for 3 seconds.
- ☐ 4 Place on the thermal cycler and run the HYB program.
- ☐ 5 Incubate at 58°C for 90 minutes to 24 hours.

Capture Hybridized Probes

- ☐ 1 Combine the following volumes to prepare Elution Master Mix.
  - EE1 (28.5 µl)
  - HP3 (1.5 µl)
- ☐ 2 Thoroughly pipette Elution Master Mix, and then set aside.
- ☐ 3 Centrifuge the PCR plate at 280 × g for 10 seconds.
- ☐ 4 Add 62.5 µl SMB.
- ☐ 5 Pipette until resuspended.
- ☐ 6 Place in the 58°C thermal cycler for 15 minutes.
- ☐ 7 Immediately do as follows.
  - ☐ a Centrifuge at 280 × g for 10 seconds.
  - ☐ b Place on the magnetic stand until liquid is clear.
- ☐ 8 Remove and discard supernatant.
- ☐ 9 Remove from the magnetic stand.
- ☐ 10 Add 50 µl preheated EEW.
- ☐ 11 Shake at 2400 rpm for 4 minutes.
- ☐ 12 Return unused EEW to the microheating system.
- ☐ 13 Return the plate to the 58°C thermal cycler for 5 minutes.
- ☐ 14 Immediately do as follows.
  - ☐ a Centrifuge at 280 × g for 3 seconds.
  - ☐ b Place on the magnetic stand until liquid is clear.
- ☐ 15 Remove and discard supernatant.
- ☐ 16 Remove from the magnetic stand.
- ☐ 17 Add 50 µl preheated EEW.
- ☐ 18 Shake at 2000 rpm for 1 minute.
- ☐ 19 Return unused EEW to the microheating system.
- ☐ 20 Return the plate to the 58°C thermal cycler for 5 minutes.
- ☐ 21 Immediately do as follows.
  - ☐ a Centrifuge at 280 × g for 3 seconds.
  - ☐ b Place on the magnetic stand until liquid is clear.
- ☐ 22 Remove and discard supernatant.
- ☐ 23 Repeat steps 16–22.
- ☐ 24 Remove from the magnetic stand.
- ☐ 25 Add 50 µl preheated EEW.
- ☐ 26 Shake at 2000 rpm for 1 minute.
- ☐ 27 Centrifuge at 280 × g for 3 seconds.
- ☐ 28 Transfer 50 µl resuspended bead solution.
- ☐ 29 Seal and centrifuge at 280 × g for 3 seconds.
- ☐ 30 Return to the 58°C thermal cycler for 5 minutes.
- ☐ 31 Immediately place on the magnetic stand until liquid is clear.
- ☐ 32 Remove and discard supernatant.
- ☐ 33 Remove and discard residual EEW.
- ☐ 34 Thoroughly pipette Elution Master Mix.
- ☐ 35 Remove from the magnetic stand.
- ☐ 36 Add 23 µl Elution Master Mix.
- ☐ 37 Shake at 2600 rpm for 1 minute.
- ☐ 38 Incubate at room temperature for 2 minutes.
- ☐ 39 Centrifuge at 280 × g for 10 seconds, and then unseal.
- ☐ 40 Place on the magnetic stand until liquid is clear.
- ☐ 41 Transfer 21 µl supernatant.
- ☐ 42 Add 4 µl ET2.
- ☐ 43 Shake at 2000 rpm for 1 minute.

SAFE STOPPING POINT

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

Amplify Enriched Library

- ☐ 1 Centrifuge the sealed plate at 280 × g for 10 seconds.
- ☐ 2 Add 5 µl PPC.
- ☐ 3 Add 20 µl EPM.
- ☐ 4 Shake at 2000 rpm for 1 minute.
- ☐ 5 Centrifuge at 280 × g for 10 seconds.
- ☐ 6 Place on the thermal cycler and run the AMP program.

Clean Up Enriched Library

- ☐ 1 Centrifuge at 280 × g for 10 seconds.
- ☐ 2 Add 90 µl AMPure XP.
- ☐ 3 Shake at 2200 rpm for 1 minute.
- ☐ 4 Incubate at room temperature for 5 minutes.
- ☐ 5 Centrifuge at 280 × g for 10 seconds, and then unseal.
- ☐ 6 Place on the magnetic stand until liquid is clear.
- ☐ 7 Remove and discard supernatant.
- ☐ 8 Wash beads as follows.
  - ☐ a Add 175 µl fresh 80% EtOH.
  - ☐ b Wait 30 seconds.
  - ☐ c Remove and discard supernatant.
- ☐ 9 Wash beads a **second** time.
- ☐ 10 Remove residual EtOH.
- ☐ 11 Air-dry on the magnetic stand for 2 minutes.
- ☐ 12 Remove from the magnetic stand.
- ☐ 13 Add 32 µl RSB.
- ☐ 14 Shake at 2600 rpm for 1 minute.
- ☐ 15 Incubate at room temperature for 2 minutes.
- ☐ 16 Centrifuge at 280 × g for 10 seconds, and then unseal.
- ☐ 17 Place on the magnetic stand until liquid is clear.
- ☐ 18 Transfer 30 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 Enriched Library

- ☐ 1 Check the enriched library:
  - ▶ Analyze 1 µl enriched library with the Qubit dsDNA HS Assay kit.
  - ▶ Analyze 1 µl enriched library with the Agilent 2100 Bioanalyzer System and a DNA 1000 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	20	0.8
NovaSeq 6000	0.6	120

- ☐ 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.