AmpliSeq for Illumina Custom and Community RNA Panels Checklist

Quantify and Dilute RNA

- ☐ 1 Quantify RNA using a fluorometric method.
- ☐ 2 If enough RNA is available, dilute to an intermediate concentration as follows.
  - ☐ a Dilute to a concentration of ~20–50 ng/µl using nuclease-free water.
  - ☐ b Requantify the diluted RNA.
- ☐ 3 Dilute RNA to desired final concentration.

Reverse Transcribe RNA

- ☐ 1 For one sample, combine the following volumes in one well of a 96-well PCR plate. For multiple samples, combine the following reagents except RNA in a 1.5 ml tube to prepare master mix.
  - 5X AmpliSeq cDNA Reaction Mix (2 µl)
  - 10X AmpliSeq RT Enzyme Mix (1 µl)
  - **[Optional]** AmpliSeq ERCC RNA Spike-In Mix for Illumina as follows.

Total RNA Input (ng)	Volume (µl)
10	1 (1:5000 dilution)
20	2 (1:5000 dilution)
50	1 (1:1000 dilution)
100	2 (1:1000 dilution)

  - Total RNA (1–100 ng per pool) (≤ 7 µl)
  - Nuclease-free water (to 10 µl)
- ☐ 2 Seal the plate.
- ☐ 3 Vortex thoroughly, and then centrifuge briefly.
- ☐ 4 Place on the thermal cycler, cover with a compression pad (if applicable), and run the RT program.

SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 10°C for up to 16 hours. For longer durations, store at -25°C to -15°C.

Amplify cDNA Targets (One Primer Pool)

- ☐ 1 Briefly centrifuge the plate to collect contents.
- ☐ 2 Add the following volumes per sample to each well.
  - 5X AmpliSeq HiFi Mix (4 µl) (red cap)
  - 5X AmpliSeq Custom RNA Panel or AmpliSeq Custom RNA Fusion Panel (4 µl) (cap color varies)
  - **[Optional]** AmpliSeq ERCC RNA Companion Panel for Illumina (1 µl)
  - Nuclease-free water (2 µl)
- ☐ 3 Pipette to mix.
- ☐ 4 Seal the plate, and then centrifuge briefly.
- ☐ 5 Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP_RNA program.
- ☐ 6 Proceed to *Partially Digest Amplicons* on page 2.

SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 10°C overnight or store at -25°C to -15°C.

Amplify cDNA Targets (Two Primer Pools)

- ☐ 1 **[Optional]** If using AmpliSeq ERCC RNA Companion Panel for Illumina, do as follows.
  - ☐ a For each sample, combine the following volumes in a 1.5 ml tube:
    - 20X AmpliSeq ERCC RNA Companion Panel for Illumina (0.5 µl)
    - 2X AmpliSeq RNA Panel Pool 1 (cap color varies) (5 µl)
  - ☐ b Pipette to mix, and then centrifuge briefly.
- ☐ 2 Briefly centrifuge the plate to collect contents.
- ☐ 3 Add the following volumes per sample to each well .
  - 5X AmpliSeq HiFi Mix (4.5 µl) (red cap)
  - Nuclease-free water (3.5 µl)
- ☐ 4 Pipette to mix.
- ☐ 5 Seal the plate, and then centrifuge briefly.
- ☐ 6 If not using AmpliSeq ERCC RNA Companion Panel for Illumina, transfer each sample to a PCR plate as follows.
  - ☐ a Transfer 8 µl master mix to two wells.
  - ☐ b Add 2 µl 5X AmpliSeq Custom RNA or AmpliSeq Custom RNA Fusion Panel Pool 1 (cap color varies) to the first well.
  - ☐ c Add 2 µl 5X AmpliSeq Custom RNA or AmpliSeq Custom RNA Fusion Panel Pool 2 (cap color varies) to the second well.
- ☐ 7 If using AmpliSeq ERCC RNA Companion Panel for Illumina, transfer each sample to a PCR plate as follows.
  - ☐ a Transfer 8 µl master mix to two wells
  - ☐ b Add 2.5 µl 5X AmpliSeq Custom RNA or AmpliSeq Custom RNA Fusion Panel Pool 1 (cap color varies) plus AmpliSeq ERCC RNA Companion Panel for Illumina mix to the first well.
  - ☐ c Add 2 µl 5X AmpliSeq Custom RNA or AmpliSeq Custom RNA Fusion Panel Pool 2 (cap color varies) to the second well.
- ☐ 8 Seal the plate.
- ☐ 9 Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP_RNA program.

SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 10°C overnight or store at -25°C to -15°C.

Partially Digest Amplicons

- ☐ 1 Briefly centrifuge to collect contents.
- ☐ 2 **[Two primer pools]** For each sample, combine the 10 µl target amplification reaction from the sample well containing pool 2 into the sample well containing pool 1.
- ☐ 3 Add 2 µl FuPa Reagent (brown cap) to each target amplification reaction.
- ☐ 4 Vortex briefly, and then centrifuge briefly.
- ☐ 5 Place on the thermal cycler, cover with a compression pad (if applicable), and run the preprogrammed FUPA program.

SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 10°C for up to 1 hour. For longer periods, store at -25°C to -15°C.

Ligate Indexes

- ☐ 1 Briefly centrifuge the library plate to collect contents.
- ☐ 2 Add the following volumes ***in the order listed*** to each well.

Reagent	Volume (µl)
Switch Solution (yellow cap)	4
AmpliSeq CD Indexes or UD Indexes for Illumina	2
DNA Ligase (blue cap)	2

- ☐ 3 Seal the library plate.
- ☐ 4 Vortex briefly, and then centrifuge briefly.
- ☐ 5 Place on the thermal cycler, cover with a compression pad (if applicable), and run the LIGATE program.
- ☐ 6 If the index plate contains unused indexes, seal the plate and return to storage.

Clean Up Library

- ☐ 1 Briefly centrifuge the plate to collect contents.
- ☐ 2 Add 30 µl AMPure XP beads to each library.
- ☐ 3 Vortex briefly.
- ☐ 4 Inspect each well to make sure that the mixture is homogeneous.
- ☐ 5 Centrifuge briefly.
- ☐ 6 Incubate at room temperature for 5 minutes.
- ☐ 7 Place on a magnetic stand until the mixture is clear.
- ☐ 8 Remove and discard supernatant.
- ☐ 9 Wash beads two times as follows.
  - ☐ a Add 150 µl freshly prepared 70% ethanol.
  - ☐ b Incubate at room temperate until the solution is clear (~30 seconds).
  - ☐ c Without disturbing the pellet, remove and discard supernatant.
- ☐ 10 Centrifuge briefly.
- ☐ 11 Place on the magnetic stand.
- ☐ 12 Immediately remove all residual EtOH as follows.
  - ☐ a Use a 20 µl pipette to remove residual EtOH.
  - ☐ b Air-dry on the magnetic stand.
  - ☐ c Inspect each well to make sure that the EtOH has evaporated.
  - ☐ d If EtOH remains, continue to air-dry until EtOH is no longer visible.
- ☐ 13 If you are using the AmpliSeq Library Equalizer for Illumina, proceed to *Equalize Libraries* on page 5. Otherwise, continue to *Amplify Library* on page 3.

Amplify Library

- ☐ 1 For each reaction, combine the following volumes.

Reagent	Volume (µl)
1X Lib Amp Mix (black cap)	45
10X Library Amp Primers (pink cap)	5

- ☐ 2 Vortex briefly, and then centrifuge briefly.
- ☐ 3 Remove the plate from the magnetic stand.
- ☐ 4 Add 50 µl Amplification Master Mix to each library well.
- ☐ 5 Vortex briefly, and then centrifuge briefly.
- ☐ 6 Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP_7 program.

SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 10°C for up to 24 hours. For longer durations, store at -25°C to -15°C.

Perform Second Cleanup

- ☐ 1 Briefly centrifuge the plate to collect contents.
- ☐ 2 Add 25 µl AMPure XP beads to each well.
- ☐ 3 Vortex briefly, and then centrifuge briefly.
- ☐ 4 Incubate at room temperature for 5 minutes.
- ☐ 5 Place the plate on a magnetic stand until the liquid is clear.
- ☐ 6 Transfer the entire supernatant (~75 µl), which contains the desired amplicon library, to a new plate.
- ☐ 7 Add 60 µl AMPure XP beads.
- ☐ 8 Vortex briefly, and then centrifuge briefly.
- ☐ 9 Incubate at room temperature for 5 minutes.
- ☐ 10 Place on the magnetic stand until the liquid is clear.
- ☐ 11 Without disturbing the beads, remove and discard supernatant.
- ☐ 12 Wash beads two times as follows.
  - ☐ a Add 150 µl freshly prepared 70% ethanol.
  - ☐ b Incubate at room temperate until the solution is clear (~30 seconds).
  - ☐ c Without disturbing the pellet, remove and discard supernatant.
- ☐ 13 Use a 20 µl pipette to remove residual EtOH .
- ☐ 14 Discard unused 70% EtOH.
- ☐ 15 Air-dry on the magnetic stand for 5 minutes.
- ☐ 16 Remove from the magnetic stand.
- ☐ 17 Add 30 µl Low TE to each well.
- ☐ 18 Vortex briefly, and then centrifuge briefly.
- ☐ 19 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
- ☐ 20 Transfer 27 µl supernatant to a new plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 30 days.

Check Libraries

- ☐ 1 Place the plate on the magnetic stand.
- ☐ 2 Assess library quality.
- ☐ 3 Quantify the library.

Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

For sequencing, Illumina recommends setting up a paired-end run of 151 cycles per read (2 × 151 run format).

☐ 1 Calculate the molarity value of the library or pooled libraries.

Using the molarity value, calculate the volumes of Low TE and library needed to dilute libraries to the starting concentration.

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
iSeq 100 System	2	50
MiniSeq System	2	1.1–1.9
MiSeq System (v3 reagents)	2	7–9
NextSeq 550 and NextSeq 500	2	1.1–1.9
NextSeq 2000	2	750

Dilute libraries using Low TE:

- **Libraries quantified as a pool**—Dilute the pool to the starting concentration.
- **Libraries quantified individually**—Dilute each library to the starting concentration. Add 10 µl each diluted library to a tube.

Dilute to the final loading concentration.

The final loading concentrations are a starting point and general guideline. Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

Equalize Libraries

☐ 1 Use the following steps to normalize library concentration without quantification using the AmpliSeq Library Equalizer for Illumina.

Amplify Library

- ☐ 1 Remove the plate with purified libraries from the magnetic stand.
- ☐ 2 For each reaction, combine the following volumes.

Reagent	Volume (µl)
1X Lib Amp Mix (black cap)	45
10X Library Amp Primers (pink cap)	5

- ☐ 3 Vortex briefly, and then centrifuge briefly.
- ☐ 4 Add 50 µl Amplification Master Mix to each library well.
- ☐ 5 Place on the thermal cycler, cover with a compression pad (if applicable), and run the EQUAL program.

Wash Equalizer Beads

- ☐ 1 For each reaction, combine the following volumes:
  - Equalizer Beads (7 µl)
  - Equalizer Wash Buffer (14 µl)
- ☐ 2 Pipette to mix.
- ☐ 3 Place on the magnetic stand until liquid is clear.
- ☐ 4 Remove and discard all supernatant.
- ☐ 5 Remove from the magnetic stand.
- ☐ 6 For each reaction, add 7 µl Equalizer Wash Buffer. Pipette to resuspend.

Add Equalizer Capture

- ☐ 1 Briefly centrifuge the library plate to collect contents, and then unseal.
- ☐ 2 Place on the magnetic stand until liquid is clear.
- ☐ 3 Transfer 45 µl supernatant to a new plate.
- ☐ 4 Add 10 µl Equalizer Capture.
- ☐ 5 Seal the plate, vortex to mix, and then briefly centrifuge to collect contents.
- ☐ 6 Incubate at room temperature for 5 minutes.

Perform Second Cleanup

- ☐ 1 Unseal the plate.
- ☐ 2 Vortex or pipette washed Equalizer Beads to mix.
- ☐ 3 Add 6 µl Equalizer Beads.
- ☐ 4 Seal the plate, vortex thoroughly, and then centrifuge briefly.
- ☐ 5 Incubate at room temperature for 5 minutes.
- ☐ 6 Place on the magnetic stand until liquid is clear.
- ☐ 7 Unseal the plate.
- ☐ 8 Remove and discard all supernatant.

Elute Library

- ☐ 1 Remove the plate from the magnetic stand.
- ☐ 2 Add 30 µl Equalizer Elution Buffer.
- ☐ 3 Seal the plate, vortex thoroughly, and then centrifuge briefly.
- ☐ 4 Elute the library by incubating on a thermal cycler at 45°C for 5 minutes.
- ☐ 5 Place on the magnetic stand until liquid is clear.
- ☐ 6 Unseal the plate.
- ☐ 7 Transfer 27 µl supernatant to a new plate.

SAFE STOPPING POINT

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

Denature and Dilute Libraries

- ☐ 1 Denature and dilute libraries for loading on the sequencing instrument you are using.