AmpliSeq for Illumina On-Demand, Custom and Community DNA Panels Checklist

Quantify and Dilute DNA

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

Number of Pools	DNA Concentration (ng/µl)	Diluted DNA Volume (µl)	Total DNA Input (ng)
1	2	5	10
2	4	5	20
3	6	5	30
4	8	5	40

Amplify DNA Targets (One Primer Pool)

- ☐ 1 **[One sample]** Add the following volumes to one well of a PCR plate.
- ☐ 2 **[Multiple samples]** Prepare master mix as follows.
  - ☐ a Combine all the following reagents except DNA in a 1.5 ml tube.
  - ☐ b Pipette or vortex briefly, and then centrifuge briefly.
  - ☐ c Dispense into each well, and then add DNA.

Reagent	Volume (µl)
5X AmpliSeq HiFi Mix (red cap)	4
2X AmpliSeq Custom DNA Panel (cap color varies)	10
[Optional] 20X AmpliSeq Sample ID Panel for Illumina	1
DNA (1–100 ng)	≤ 6 (≤ 5 if using AmpliSeq Sample ID Panel for Illumina)
Nuclease-free water	To reach total required volume
Total Volume	**20**

- ☐ 3 Pipette to mix, seal the plate, and then centrifuge briefly.
- ☐ 4 Place on the preprogrammed thermal cycler, cover with a compression pad (if applicable), and run the AMP_DNA program.
- ☐ 5 Proceed to *Partially Digest Amplicons* on page 3

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.

Amplify DNA Targets (Two Primer Pools)

- ☐ 1 **[Optional]** If using AmpliSeq Sample ID Panel for Illumina, do as follows.
  - ☐ a For each sample, combine the following volumes in a 1.5 ml tube:
    - 20X AmpliSeq Sample ID Panel for Illumina (0.5 µl)
    - 2X AmpliSeq DNA Panel Pool 1 (cap color varies) (5 µl)
  - ☐ b Pipette to mix, and then centrifuge briefly.
- ☐ 2 For each sample, combine the following volumes in a 1.5 ml tube:

Reagent	Volume (µl)
5X AmpliSeq HiFi Mix (red cap)	5
DNA (2–200 ng)	≤ 7.5
Nuclease-free water	To reach total required volume
Total Volume	**12.5**

- ☐ 3 Pipette to mix, and then centrifuge briefly.
- ☐ 4 If not using AmpliSeq Sample ID Panel for Illumina, transfer each sample to a PCR plate as follows.
  - ☐ a Transfer 5 µl master mix to two wells.
  - ☐ b Add 5 µl 2X AmpliSeq DNA Panel Pool 1 (cap color varies) to the first well.
  - ☐ c Add 5 µl 2X AmpliSeq DNA Panel Pool 2 (cap color varies) to the second well.
- ☐ 5 If using AmpliSeq Sample ID Panel for Illumina, transfer each sample to a PCR plate as follows.
  - ☐ a Transfer 5 µl master mix to two wells.

- ☐ b Add 5.5 µl 2X AmpliSeq DNA Panel Pool 1 (cap color varies) plus AmpliSeq Sample ID Panel for Illumina mix to the first well.
- ☐ c Add 5 µl 2X AmpliSeq DNA Panel Pool 2 (cap color varies) to the second well.

☐ 6 Pipette to mix, seal the plate, and then centrifuge briefly.

☐ 7 Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP_DNA program.

☐ 8 Proceed to *Partially Digest Amplicons* on page 3

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.

Amplify DNA Targets (Three Primer Pools)

☐ 1 **[Optional]** If using AmpliSeq Sample ID Panel for Illumina, do as follows.

- ☐ a For each sample, combine the following volumes in a 1.5 ml tube:
  - ▶ 20X AmpliSeq Sample ID Panel for Illumina (0.5 µl)
  - ▶ 2X AmpliSeq Custom DNA Panel Pool 1 (cap color varies) (5 µl)
- ☐ b Pipette to mix, and then centrifuge briefly.

☐ 2 For each sample, combine the following volumes in a 1.5 ml tube:

Reagent	Volume (µl)
5X AmpliSeq HiFi Mix (red cap)	7
DNA (3–300 ng)	≤ 10.5
Nuclease-free water	To reach total required volume
Total Volume	**17.5**

☐ 3 Pipette to mix, and then centrifuge briefly.

☐ 4 If not using AmpliSeq Sample ID Panel for Illumina, transfer each sample to a PCR plate as follows.

- ☐ a Transfer 5 µl master mix to three wells of the plate.
- ☐ b Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 1 (cap color varies) to the first well.
- ☐ c Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 2 (cap color varies) to the second well.
- ☐ d Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 3 (cap color varies) to the third well.

☐ 5 If using AmpliSeq Sample ID Panel for Illumina, transfer each sample to a PCR plate as follows.

- ☐ a Transfer 5 µl master mix to three wells of the platex.
- ☐ b Add 5.5 µl 2X AmpliSeq Custom DNA Panel Pool 1 (cap color varies) plus AmpliSeq Sample ID Panel for Illumina mix to the first well.
- ☐ c Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 2 (cap color varies) to the second well.
- ☐ d Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 3 (cap color varies) to the third well.

☐ 6 Pipette to mix, seal the plate, and then centrifuge briefly.

☐ 7 Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP_DNA program.

☐ 8 Proceed to *Partially Digest Amplicons* on page 3

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.

Amplify DNA Targets (Four Primer Pools)

☐ 1 **[Optional]** If using AmpliSeq Sample ID Panel for Illumina, do as follows.

- ☐ a For each sample, combine the following volumes in a 1.5 ml tube:
  - ▶ 20X AmpliSeq Sample ID Panel for Illumina (0.5 µl)
  - ▶ 2X AmpliSeq Custom DNA Panel Pool 1 (cap color varies) (5 µl)
- ☐ b Pipette to mix, and then centrifuge briefly.

☐ 2 For each sample, combine the following volumes in a 1.5 ml tube.

Reagent	Volume (µl)
5X AmpliSeq HiFi Mix (red cap)	9
DNA (4–400 ng)	≤ 13.5
Nuclease-free water	To reach total required volume
Total Volume	**22.5**

☐ 3 Pipette to mix, and then centrifuge briefly.

- ☐ 4 If not using AmpliSeq Sample ID Panel for Illumina, transfer each sample to a PCR plate as follows.
  - ☐ a Transfer 5 µl master mix to four wells of the plate.
  - ☐ b Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 1 (cap color varies) to the first well.
  - ☐ c Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 2 (cap color varies) to the second well.
  - ☐ d Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 3 (cap color varies) to the third well.
  - ☐ e Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 4 (cap color varies) to the fourth well.
- ☐ 5 If using AmpliSeq Sample ID Panel for Illumina, transfer each sample to a PCR plate as follows.
  - ☐ a Transfer 5 µl master mix to four wells of the plate.
  - ☐ b Add 5.5 µl 2X AmpliSeq Custom DNA Panel Pool 1 (cap color varies) plus AmpliSeq Sample ID Panel for Illumina mix to the first well.
  - ☐ c Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 2 (cap color varies) to the second well.
  - ☐ d Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 3 (cap color varies) to the third well.
  - ☐ e Add 5 µl 2X AmpliSeq Custom DNA Panel Pool 4 (cap color varies) to the fourth well.
- ☐ 6 Pipette to mix, seal the plate, and then centrifuge briefly.
- ☐ 7 Place on the thermal cycler, cover with a compression pad (if applicable), and run the AMP_DNA 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.

Partially Digest Amplicons

- ☐ 1 Briefly centrifuge to collect contents.
- ☐ 2 **[Multiple primer pools]** For each sample, combine the two, three, or four 10 µl target amplification reactions into the well containing pool 1, without changing tips.
- ☐ 3 Add the appropriate volume of FuPa Reagent (brown cap) to each target amplification reaction.

Number of Primer Pools	FuPa Volume (µl)
1 or 2	2
3	3
4	4

- ☐ 4 Vortex briefly, and then centrifuge briefly.
- ☐ 5 Place on the thermal cycler, cover with a compression pad (if applicable), and run the 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)		
	One or Two Pools	Three Pools	Four Pools
Switch Solution (yellow cap)	4	6	8
AmpliSeq CD Indexes or UD Indexes for Illumina	2	3	4
DNA Ligase (blue cap)	2	3	4

☐ 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.

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.

Clean Up Library

☐ 1 Briefly centrifuge the plate to collect contents.

☐ 2 Add the appropriate volume of AMPure XP beads to each library.

Number of Primer Pools	Bead Volume (µl)
1 or 2	30
3	45
4	60

☐ 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 6. Otherwise, continue to *Amplify Library* on page 1.

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. Keep the plate on the stand while performing normalization and pooling.
- ☐ 2 Assess library quality.
- ☐ 3 Quantify the library.

Dilute Libraries to the Starting Concentration

- ☐ 1 Calculate the molarity value of the library or pooled libraries.
- ☐ 2 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

- ☐ 3 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.
- ☐ 4 Dilute to the final loading concentration.

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.