Ion AmpliSeq™ Library Kit Plus

DNA Library Preparation with 1- or 2-Pool Panels using the Ion Library Equalizer™ Kit

Catalog Numbers 4488990, A35907, A38875

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Note: For safety and biohazard guidelines, see the "Safety" appendix in the following product documentation: *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

This quick reference is intended as a benchtop reference for experienced users of the Ion AmpliSeq™ Library Kit Plus who are using the Ion Library Equalizer™ Kit to normalize the concentration of libraries prepared from DNA using 1- or 2-pool panels. See the *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003) for detailed instructions, including protocols for 3- and 4-pool panels, and libraries from RNA.

Set up DNA target amplification reactions

Set up DNA target amplification reactions using one of the following procedures, depending on the number of primer pools in your Ion AmpliSeq™ panel.

Prepare DNA target amplification reactions—1 primer pool

For DNA panels with 1 primer pool, target amplification reactions can be assembled directly in a 96-well plate, one well per sample. Prepare the reactions based on whether you are using a 2X primer pool panel or a 5X primer pool panel. For multiple reactions, prepare a master mix without sample DNA.

If using the Ion AmpliSeq™ Direct FFPE DNA Kit, see *Ion AmpliSeq™ Direct FFPE DNA Kit User Guide* (Pub No. MAN0014881)

1. If using the Ion AmpliSeq™ Direct FFPE DNA Kit, add up to the maximum volume of DNA indicated in the following table to each well to achieve the maximum amount of DNA in the reaction.

   For sample DNA prepared otherwise, add up to 100 ng of DNA to each well.

Component	Volume
2X single-primer pool panel	
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
2X Ion AmpliSeq™ Primer Pool	10 µL
DNA (1–100 ng), or Direct FFPE DNA preparation	≤6 µL
Nuclease-free water	to 20 µL
5X single-primer pool panel	
5X Ion AmpliSeq™ HiFi Mix (red cap)	4 µL
5X Ion AmpliSeq™ Primer Pool	4 µL
DNA (1–100 ng), or Direct FFPE DNA preparation	≤12 µL
Nuclease-free water	to 20 µL

2. Add the remaining reagents to the wells in the volumes that are indicated in the table. Bring the total volume to 20 µL.
3. Seal the plate with a MicroAmp™ Clear Adhesive Film.
4. Place a MicroAmp™ Optical Film Compression Pad on the plate, then load the plate into the thermal cycler.

Proceed to "Amplify the targets" on page 2.

Prepare DNA target amplification reactions—2 primer pools

If you are using a DNA panel with 2 primer pools, set up two 10-µL amplification reactions, then combine them after target amplification to yield a total volume of 20 µL. Prepare the reactions based on whether you are using a 2X primer pool panel or a 5X primer pool panel.

1. For DNA panels with 2 primer pools, use the following table to prepare for each sample a target amplification master mix without primers in a 1.5-mL tube.

   If using the Ion AmpliSeq™ Direct FFPE DNA Kit, remove up to the maximum volume indicated in the table from the lower aqueous phase of the well. Add to the target amplification reaction master mix.

Component	Volume
2X 2-primer pool panel	
5X Ion AmpliSeq™ HiFi Mix (red cap)	5 µL
DNA (2–100 ng), or Direct FFPE DNA preparation	≤7.5 µL
Nuclease-free water	to 12.5 µL
5X 2-primer pool panel	
5X Ion AmpliSeq™ HiFi Mix (red cap)	4.5 µL
DNA (2–100 ng), or Direct FFPE DNA preparation	≤13.5 µL
Nuclease-free water	to 18 µL

2. Mix thoroughly by pipetting up and down 5 times, then transfer sample-specific master mixes to 2 wells of a 96-well PCR plate.
   - For 2X primer pools, transfer 5 µL of master mix into 2 wells. Add 5 µL of primer pool 1 into the first well, and 5 µL of primer pool 2 to the second well.
   - For 5X primer pools, transfer 8 µL of master mix into 2 wells. Add 2 µL of primer pool 1 into the first well, and 2 µL of primer pool 2 to the second well.
3. Seal the plate with a MicroAmp™ Clear Adhesive Film.
4. Place a MicroAmp™ Optical Film Compression Pad on the plate, then load the plate into the thermal cycler.

Amplify the targets

1. To amplify target regions, run the following program.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	2 minutes
Cycle Set the cycle number according to the following tables.	Denature	99°C	15 seconds
	Anneal and extend	60°C	4/8/16 minutes Set the time according to the following tables.
Hold	—	10°C	Hold

   The following amplification cycle parameters are recommended based on 10-ng DNA input.

Primer pairs per pool	Recommended number of amplification cycles (10 ng DNA, 3,000 copies)		Anneal/extend time
	High quality DNA	Low quality DNA (FFPE DNA or cfDNA)	
12–24	21	24	4 minutes
25–48	20	23	4 minutes
49–96	19	22	4 minutes
97–192	18	21	4 minutes
193–384	17	20	4 minutes
385–768	16	19	4 minutes
769–1,536	15	18	8 minutes
1,537–3,072	14	17	8 minutes
3,073–6,144	13	16	16 minutes
6,145–24,576	12	15	16 minutes

Exceptions can be made to the recommended parameters for the following panels.

Ion AmpliSeq™ panel	Primer pairs per pool	Description of change
Ion AmpliSeq™ Comprehensive Cancer Panel (Cat. No. 4477685)	~4,000	Use 8 minutes anneal/extend time instead of 16 minutes
Ion AmpliSeq™ panels using a 375-bp amplicon design	—	Add 4 minutes to the anneal/extend time recommended in the table

2. *(Optional)* Adjust the cycle number from the preceding table for lower or higher DNA input.

Amount of DNA starting material	Adjustment to cycle number
1 ng (300 copies)	+3
10 ng (3,000 copies)	0
100 ng (30,000 copies)	–3

STOPPING POINT Target amplification reactions can be stored at 10°C overnight on the thermal cycler. For longer term, store at –20°C.

Combine target amplification reactions (2-pool DNA panels only)

1. For one primer pool panel, tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents, then remove the plate seal.
2. Carefully remove the plate seal.
3. For each sample, combine the 10-µL target amplification reactions. The total volume for each sample should be ~20 µL.

Proceed to step 2 of "Partially digest amplicons".

Partially digest amplicons

IMPORTANT! FuPa Reagent is viscous. Pipet slowly and mix thoroughly. Perform this step on ice or on a cold block, then quickly proceed to incubation.

1. For one primer pool panel, tap the plate gently on a hard flat surface, or briefly centrifuge to collect the contents, then remove the plate seal.
2. Add 2 µL of FuPa Reagent (brown cap) to each amplified sample. The total volume is ~22 µL.
3. Seal the plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.
4. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate in the thermal cycler, then run the following program.

Temperature	Time
50°C	10 minutes[1]
55°C	10 minutes[1]
60°C	20 minutes
10°C	Hold (for up to 1 hour)

[1] Increase to 20 minutes for panels over 1,536 primer pairs.

5. Tap the plate gently on a hard flat surface, or briefly centrifuge to collect the contents.

STOPPING POINT Store plate at –20°C for longer periods.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single chip, you *must* ligate a different barcode adapter to each library. DNA and RNA libraries from the same sample also require different barcodes.

Ion Torrent™ Dual Barcode Adapters are provided at the appropriate concentration. No further handling is necessary.

IonCode™ Adapters are provided at the appropriate concentration and include forward and reverse adapters in a single well. No further handling is necessary.

Ion Xpress™ adapters require handling and dilution as described in the *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

Perform the ligation reaction

1. If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.
2. Briefly centrifuge the plate to collect the contents.
3. Carefully remove the plate seal, then add the following components in the order that is listed to each well containing digested amplicons. If preparing multiple non-barcoded libraries, a master mix of Switch Solution and adapters can be combined before addition.

IMPORTANT! Add the DNA Ligase last. Do not combine DNA Ligase and adapters before adding to digested amplicons.

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Adapters (Ion Torrent™ Dual Barcode Adapters, IonCode™ Barcode Adapters, *or* diluted Ion Xpress™ barcode adapter mix (for barcoded libraries))	2 µL
3	DNA Ligase (blue cap)	2 µL
Total volume		**~30 µL**

4. Seal the plate with a new MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
5. Place a MicroAmp™ Optical Film Compression Pad on the plate, load in the thermal cycler, then run the following program.

Temperature	Time
22°C	30 minutes
68°C	5 minutes
72°C	5 minutes
10°C	Hold (for up to 24 hours)

Purify the library

IMPORTANT!

- The recommended product for purification is MagMAX™ Pure Bind Beads. Another option is Agencourt™ AMPure™ XP Reagent.
- MagMAX™ Pure Bind Beads stored at room temperature can be used immediately after mixing thoroughly to disperse the beads before use. If using Agencourt™ AMPure™ XP Reagent or MagMAX™ Pure Bind Beads stored at 4°C, bring to room temperature for 30 minutes before use, then mix thoroughly to disperse the beads before use.
- Pipet the solution slowly.
- Do NOT substitute a Dynabeads™-based purification reagent for the MagMAX™ Pure Bind Beads.

1. Briefly centrifuge the plate to collect the contents.
2. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of MagMAX™ Pure Bind Beads to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.
5. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet to wash the beads. Carefully remove, then discard the supernatant without disturbing the pellet.
6. Repeat step 5 for a second wash.
7. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes.

Equalize the library

Before you begin

Warm all the reagents in the Ion Library Equalizer™ Kit to room temperature. Vortex and centrifuge all reagents before use.

Amplify the library

1. Remove the plate with purified libraries from the plate magnet, then add 50 µL of 1X Library Amp Mix (black cap) and 2 µL of Equalizer™ Primers (pink cap), or 2 µL of 25X Library Amp Primers, to each bead pellet.
2. Seal the plate with a clear adhesive film, vortex thoroughly, then centrifuge to collect droplets.
3. Place the plate back on the magnet for at least 2 minutes, then carefully transfer ~50 µL of supernatant from each well to a new well or a new plate without disturbing the pellet.
4. Seal the plate with a new clear adhesive film, place a compression pad on the plate, then load in the thermal cycler. Run the following program.

Stage	Temperature	Time
Hold	98°C	2 minutes
9 cycles	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold (up to 1 hour)

5. (Optional) If possible after thermal cycling, briefly centrifuge the plate to collect the contents.

Wash the Equalizer™ Beads *(if not previously washed)*

1. Bring the Equalizer™ Beads to room temperature, then mix thoroughly.
2. For each reaction, pipet 3 µL of beads into a clean 1.5-mL tube, then add 6 µL per reaction of Equalizer™ Wash Buffer.
3. Place the tube in a magnetic rack for 3 minutes, or until the solution is clear.
4. Carefully remove the supernatant without disturbing the pellet, then discard.
5. Remove the tube from the magnet, add 6 µL per reaction of Equalizer™ Wash Buffer, then pipet up and down to resuspend.

Add Equalizer™ Capture to the amplified library

1. Carefully remove the seal from the plate, then add exactly 10 µL of Equalizer™ Capture reagent to each library amplification reaction.

   The final equalized library concentration depends on accurate pipetting of the Equalizer™ Capture reagent.
2. Seal the plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.
3. Incubate at room temperature for 5 minutes.

(Optional) Combine captured libraries

1. Determine the number of samples to be combined based on the coverage depth tables. .

   See the *Ion AmpliSeq™ Library Kit Plus User Guide*

2. Determine the number of samples to be combined based on the coverage depth tables.

   See "Ion Chip capacities for Ion AmpliSeq™ DNA libraries sequenced at equal depths" in Appendix D of the *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

3. Carefully remove the seal from the plate, then remove and combine an equal volume (5–10 µL) of each sample into a single well or tube. Mix the combined libraries thoroughly, then transfer 60 µL to a new well. Treat the combined libraries as a single sample and proceed to the next section.

   Example 1— If 8 libraries are to be combined in a single templating and sequencing reaction, remove 7.5 µL of each library and combine them together in a new position on the 96-well plate.

   Example 2— If 384 libraries are to be combined in a single templating and sequencing reaction, remove 5 µL of each library and combine them in a 2-mL tube. Mix thoroughly, then transfer 60 µL to a new position on the 96-well plate.

   Save uncombined individual libraries for repeat analysis, if needed.

Add Equalizer™ Beads and wash

1. Gently vortex or pipet up and down to mix the washed Equalizer™ Beads.
2. If needed, carefully remove the seal from the plate, then add 6 µL of washed Equalizer™ Beads to each plate well that contains the captured library (either combined or individual).
3. Set the pipette volume to 40 µL, then pipet the mixture up and down at least 5 times to mix thoroughly.
4. Incubate at room temperature for 5 minutes.

   Check for droplets on the sides of the plate wells. If droplets are observed, seal the plate, then gently tap the plate on a hard, flat surface, or briefly centrifuge to collect the contents.

5. Place the plate in the magnet, then incubate for 2 minutes or until the solution is clear.
6. If needed, carefully remove the seal from the plate, then remove the supernatant without disturbing the pellet.
7. Add 150 µL of Equalizer™ Wash Buffer to each reaction.
8. To wash the beads, move the plate side-to-side in the two positions of the magnet.
9. With the plate still in the magnet, carefully remove, then discard the supernatant without disturbing the pellet.
10. Repeat the bead wash as described in step 7 through step 9.

    Ensure that as much wash buffer as possible is removed without disturbing the pellet.

Elute the equalized library

1. Remove the plate from the magnet, then add 100 µL of Equalizer™ Elution Buffer to each pellet.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.

   Centrifuge with enough force to collect the contents, but not the pellet beads. If beads are pelleted, vortex again and centrifuge more gently.

3. Elute the library by incubating in a thermal cycler at 32°C for 5 minutes.
4. Place the plate in the magnet, then incubate at room temperature for 5 minutes or until the solution is clear.

   The supernatant contains the equalized library at ~100 pM, which can be stored with beads for up to 1 month at 4–8°C.

Proceed to template preparation, or combine or store libraries as described in “Store libraries” on page 5.

(Optional) Combine amplicon libraries

Multiple strategies for combining libraries are available. See Appendix D, “Strategies for combining Ion AmpliSeq™ libraries”, in the *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

Store libraries

Store libraries at 4–8°C for up to 1 month. For longer lengths of time, store at –30°C to –10°C.

Limited product warranty

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: Pub. No. MAN0017004 C.0

Revision	Date	Description
C.0	17 December 2023	• Updated to correspond to the *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003) Revision D.0. • Added support for MagMAX™ Pure Bind Beads for purification.
B.0	5 November 2018	• Added Ion AmpliSeq™ Library Kit Plus 384-reaction size (Cat. No. A38875) • Support added for Ion GeneStudio™ S5 Systems • Support added for Ion Torrent™ Dual Barcode Kit 1–96 • Protocol updates
A.0	15 May 2017	New quick reference for the Ion AmpliSeq™ Library Kit Plus