Ion AmpliSeq™ Library Kit 2.0

DNA Library Preparation with 1- or 2-Pool Panels Using qPCR Quantification

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Note: For safety and biohazard guidelines, see the "Safety" appendix in the following product documentation: *Ion AmpliSeq™ Library Kit 2.0 User Guide* (Pub. No. MAN0006735). 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 Ion AmpliSeq™ Library Kit 2.0 who are performing qPCR quantification for libraries prepared from DNA using 1- or 2-pool panels. See the *Ion AmpliSeq™ Library Kit 2.0 User Guide* (Pub. No. MAN0006735) 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™ Barcode Adapters are provided at the appropriate concentration and include forward and reverse adapters in a single well. No further handling is necessary.

Ion Xpress™ Barcode Adapters require handling and dilution as described in the *Ion AmpliSeq™ Library Kit 2.0 User Guide*.

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. If you have not already done so, briefly centrifuge the plate to collect the contents, then carefully remove the plate seal.
3. Add the following components in the order 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 the contents.
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.

Quantify the unamplified library by qPCR

Elute the unamplified Ion AmpliSeq™ library, then determine the concentration by qPCR with the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802). Unamplified libraries typically have yields of 100–500 pM. After quantification, determine the dilution factor that results in a concentration of ~100 pM, which is appropriate for template preparation using an Ion template kit.

Elute and dilute the library

1. Remove the plate with purified libraries from the plate magnet, then add 50 µL of Low TE to the pellet to disperse the beads.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.
3. Incubate at room temperature for at least 2 minutes.
4. Place the plate on the magnet for at least 2 minutes.
5. Prepare a 100-fold dilution for quantification. Remove 2 µL of supernatant that contains the library, then combine with 198 µL of nuclease-free water.

Proceed immediately to “Quantify library by qPCR and calculate the dilution factor” on page 4.

Quantify library by qPCR and calculate the dilution factor

Determine the concentration of each Ion AmpliSeq™ library by qPCR with the Ion Library TaqMan™ Quantitation Kit using the following steps. Analyze each sample, standard, and negative control in duplicate 20-µL reactions.

1. Prepare three 10-fold serial dilutions of the *E. coli* DH10B Control Library (~68 pM; from the Ion Library TaqMan™ Quantitation Kit) at 6.8 pM, 0.68 pM, and 0.068 pM. Mark these tubes as standards, then use these concentrations in the qPCR instrument software.
2. Prepare reaction mixtures. For each sample, control, and standard, combine 20 µL of 2X Ion Library qPCR Master Mix and 2 µL of Ion Library TaqMan™ Quantitation Assay, 20X , then mix thoroughly. Dispense 11-µL aliquots into the wells of a PCR plate.
3. Add 9 µL of the diluted (1:100) Ion AmpliSeq™ library or 9 µL of each control dilution to each well (two wells per sample as noted before), for a total reaction volume of 20 µL.
4. Program your real-time instrument.
   a. Enter the concentrations of the control library standards.
   b. Select ROX™ Reference Dye as the passive reference dye.
   c. Select a reaction volume of 20 µL.
   d. Select FAM™ dye/MGB as the TaqMan™ probe reporter/quencher.

e. Select one of the programs in the following table. The fast cycling program was developed using the StepOnePlus™ Real-Time PCR System in fast run mode.

IMPORTANT! When quantifying libraries made from panels with 275-bp or 375-bp designs, use standard qPCR cycling. Fast cycling can result in inaccurate quantification.

Reaction plate format	Stage	Temperature	Time
Fast run mode			
48- and 96-well Fast OR 384-well Standard	Hold (UDG incubation)	50°C	2 min
	Hold (polymerase activation)	95°C	20 sec
	Cycle (40 cycles)	95°C	1 sec
		60°C	20 sec
Standard run mode			
96-well Standard	Hold (UDG incubation)	50°C	2 min
	Hold (polymerase activation)	95°C	2 min
	Cycle (40 cycles)	95°C	15 sec
		60°C	1 min

5. Following qPCR, calculate the average concentration of the undiluted Ion AmpliSeq™ library by multiplying the concentration that is determined with qPCR by 100.

6. Based on the calculated library concentration, determine the dilution that results in a concentration of ~100 pM.

7. Dilute the library to ~100 pM, combine, then proceed to template preparation, 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 2.0 User Guide* (Pub. No. MAN0006735).

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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Revision history: Pub. No. MAN0006775 E.0

Revision	Date	Description
E.0	17 December 2023	• Updated to correspond to *Ion AmpliSeq™ Library Kit 2.0 User Guide* (Pub. No. MAN0006735) Revision G.0. • Added support for MagMAX™ Pure Bind Beads for purification.
D.0	18 January 2019	• Support added for Ion 510™ and Ion 550™ Chips. • Support added for Ion GeneStudio™ S5 Systems. • Support added for Ion Torrent™ Dual Barcode Kit 1–96. • Style updates
C.0	24 May 2017	• Lower limit of gDNA added to target amplification master mixes for 2-pool panels changed from 1 ng to 2 ng. • Last two rows of the amplification cycle table merged, anneal/extension time column added to table, and anneal/extension time recommendations modified for higher plexy panels (see "Amplify the targets" on page 2).