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 *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 — single primer pool

For panels with 1 primer pool, target amplification reactions can be assembled directly in a 96-well plate.

1. For DNA panels with 1 primer pool, select the appropriate table below based on whether you are using a 2X primer pool or a 5X primer pool. Add the following components to a single well of a 96-well PCR plate. Prepare a master mix without sample DNA for multiple reactions.

   Note: 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 and add to the target amplification reaction.

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. Seal the plate with a MicroAmp™ Clear Adhesive Film, then place a MicroAmp™ Compression Pad on the plate.

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 give a volume of 20 µL.

1. For 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.

   Note: 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 and 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, then place a MicroAmp™ Compression Pad on the plate. Proceed to "Amplify the targets".

Amplify the targets

To amplify target regions, run the following program.

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

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

Cycle number recommendations in the preceding table are based on 10-ng DNA input. Adjust 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

Exceptions to the amplification parameters recommended in the parameter table

Ion AmpliSeq™ panel	Primer pairs/pool	Description of change
Ion AmpliSeq™ Pharmacogenomics Research Panel (Cat. No. A29251)	119	Add 2 amplification cycles (20 cycles instead of 18 cycles for high-quality DNA)
Ion AmpliSeq™ Comprehensive Cancer Panel (Cat. No. 4477685)	~4,000	Use 8 minute 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

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. Carefully remove the plate seal.
2. For each sample, combine the 10-µL target amplification reactions. The total volume for each sample should be ~20 µL.

Partially digest amplicons

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

1. One-primer pool panel: tap the plate gently on a hard flat surface, or centrifuge briefly to collect the contents at the bottom of the wells, 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 clear adhesive film, vortex thoroughly, then centrifuge to collect droplets.
4. Place a compression pad on the plate, load 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.

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.

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 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. Briefly centrifuge the plate to collect the contents.
3. Carefully remove the plate seal, then 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	IonCode™ Adapters *or* diluted Ion Xpress™ barcode adapter mix (for barcoded libraries) *or* Ion AmpliSeq™ Adapters (green cap, for non-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™ 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

1. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
2. Carefully remove the plate seal, then add 45 µL (1.5X sample volume) of Agencourt™ AMPure™ XP Reagent 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 suitable for template preparation using an Ion template kit.

Elute 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™ Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.

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, containing the library, then combine with 198 µL of Nuclease-free Water.

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 Ion 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 TaqMan® MasterMix and 2 µL of 20X Ion TaqMan® Assay, 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 as follows:
   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. The Ion Library TaqMan® qPCR Mix can be used on various instruments, as listed in the following table. The fast cycling program was developed using the StepOnePlus™ System in Fast 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.

Real-time PCR System	Run mode	Stage	Temp	Time
7500 Fast	Fast	Hold[1]	50°C	2 min
7900 HT 7900 HT Fast		Hold[2]	95°C	20 sec
ViiA™ 7		Cycle (40 cycles)	95°C	1 sec
StepOne™ StepOnePlus™			60°C	20 sec
QuantStudio™ 3 or 5				
7300	Standard	Hold[1]	50°C	2 min
7500		Hold[2]	95°C	2 min
7900 HT 7900 HT Fast		Cycle (40 cycles)	95°C	15 sec
ViiA™ 7			60°C	1 min
QuantStudio™ 3 or 5				

[1] UDG incubation
[2] Polymerase activation

5. Following qPCR, calculate the average concentration of the undiluted Ion AmpliSeq™ library by multiplying the concentration 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 library to ~100 pM, combine, then proceed to template preparation, or store libraries as described below.

(Optional) Combine amplicon libraries

Multiple strategies for combining libraries are available. See Appendix C, "Strategies for combining Ion AmpliSeq™ libraries", in the *Ion AmpliSeq™ Library Kit 2.0 User Guide* (Pub. No. MAN0006735).

Store libraries

Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at –20°C.

Limited product warranty

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Manufacturer: Multiple Life Technologies Corporation manufacturing sites are responsible for manufacturing the products associated with the workflow covered in this guide.

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Revision history: Pub. No. MAN0006775

Revision	Date	Description
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)
B.0	10 March 2017	• Minor clarifications made to align with Rev. D.0 of user guide • Guidance added for target amplification conditions for 375 bp amplicon designs
A.0	23 June 2016	• Added support for the Ion AmpliSeq™ Direct FFPE DNA Kit • Added support for DNA input in range of 1–100 ng • Instructions for setting up amplification reactions for Ion AmpliSeq™ panels with more than one primer pool clarified • Added support for IonCode™ Barcode Adaptors • Ligation reaction temperature conditions updated • Table of PCR programs for qPCR library quantification updated