Ion AmpliSeq™ Exome RDY Library Preparation

Using qPCR Quantification

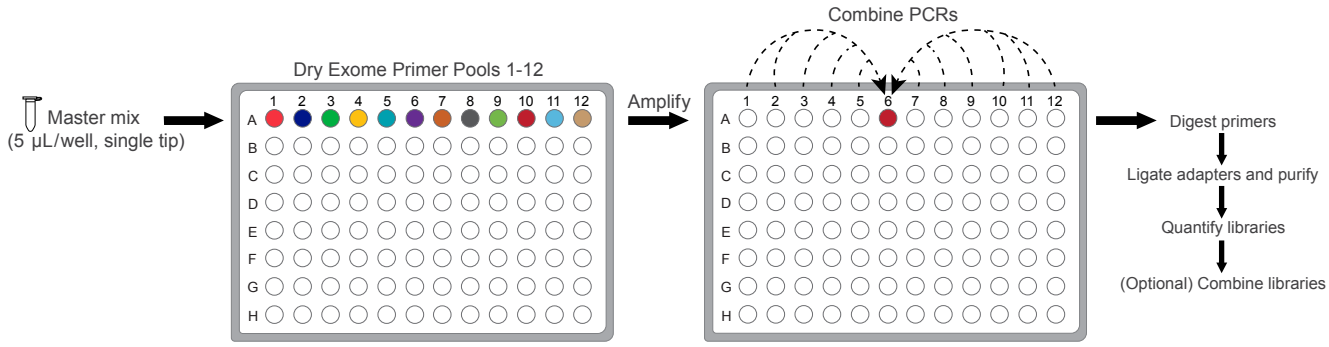
Catalog Numbers A38262, A38264

Pub. No. MAN0009809 **Rev.** C.0

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *Ion AmpliSeq™ Exome RDY Library Preparation User Guide* (Pub. No. MAN0010084). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Workflow

This guide provides a protocol for experienced users for preparing libraries using the Ion AmpliSeq™ Exome RDY Kits (Cat. Nos. A38262, A38264) for sequencing on one of the Ion GeneStudio S5 Series Sequencers, the Ion S5™/Ion S5™ XL Sequencers, or the Ion Proton™ Sequencer. For additional tips and troubleshooting, see the *Ion AmpliSeq™ Exome RDY Library Preparation User Guide* (Pub. No. MAN0010084).



Guidelines for DNA quantification

- We recommend the TaqMan® RNase P Detection Reagents Kit (Cat. No. 4316831) for quantifying amplifiable DNA. See the *Demonstrated Protocol: Sample Quantification for Ion AmpliSeq™ Library Preparation Using the TaqMan® RNAse P Detection Reagents Kit* (Pub. No. MAN0007732), available at **thermofisher.com**.
- The Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) can also be used.
- We do not recommend densitometric methods, because these methods are not specific for DNA.
- For each exome library target amplification reaction, use 50–100 ng of genomic DNA (gDNA). When DNA is not limiting, we recommend 100 ng. The maximum volume of DNA in a reaction is 56 µL.

Amplify the targets

1. For each sample, prepare a master mix:

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	14 µL
50–100 ng gDNA (non-FFPE)	≤ 56 µL
Nuclease-free Water	**to 70 µL**

2. Mix the master mix thoroughly by vortexing, or pipetting up and down 5 times, then centrifuge briefly to collect droplets.
3. Remove the seal from the Ion AmpliSeq™ Exome RDY plate.

4. For each sample, use a low volume pipettor to dispense carefully 5-µL aliquots of master mix into a single horizontal row (12 wells) of the plate without changing the tip.

   Note: A blue dye is added to the primers to help identify wells that contain dry primers. Each row in the 1×8 format (Part. No. 4489838) contains primers. Only rows "C" and "F" in the 4×2 format (Part. No. 4489840) contain primers.

5. Apply a MicroAmp™ Clear Adhesive Film, ensure a tight seal, then briefly centrifuge the plate to collect droplets.

6. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate in the thermal cycler, then run the following program for 5-µL volume.

   IMPORTANT! Use of the recommended plates, seals, compression pads, and a Thermo Fisher Scientific thermal cycler are critical for best performance.

Stage	Step	Temp	Time
Hold	Activate enzyme	99°C	2 minutes
Cycle (10 cycles)	Denature	99°C	15 seconds
	Anneal/extend	60°C	16 minutes
Hold	—	10°C	Hold

STOPPING POINT You can store PCR products at 10°C overnight. For longer lengths of time, store at –30°C to –10°C.

Partially digest amplicons

1. Briefly centrifuge the plate to collect droplets. Carefully remove the plate seal, then combine the 12 target amplification reactions for each sample (row) by transferring the reactions from wells 1–5 and 7–12 into the column 6 well, using a single tip, as shown above.

2. **Add 6 µL of FuPa Reagent** (brown cap) to each combined target amplification reaction, to bring the total volume to approximately 60 µL.

3. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

4. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate in the thermal cycler, then run the following program:

Termperature	Time
50°C	20 minutes
55°C	20 minutes
60°C	20 minutes
10°C	Hold

STOPPING POINT Hold the reactions for up to 1 hour at 10°C. For longer lengths of time, store the plate at –30°C to –10°C.

Ligate adapters to the amplicons and purify

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 below.

Ion Xpress™ Barcode Adapters adapters only: Combine and dilute adapters

For each barcode X selected, prepare a mix of Ion P1 Adapter and Ion Xpress™ Barcode X at a final dilution of 1:4 for each adapter. Store diluted adapters at –20°C.

Combine the volumes indicated in the following table. Add 6 µL of this barcode adapter mix to the ligation reaction in step 3 on the following page.

Component	Volume
Ion P1 Adapter	2 µL
Ion Xpress™ Barcode X[1]	2 µL
Nuclease-free Water	4 µL
Total	**8 µL**

[1] X = barcode selected

Set up and run 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.

   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)	12 µL
2	IonCode™ Barcode Adapters *or* diluted Ion Xpress™ Barcode Adapters mix (for barcoded libraries)	6 µL
3	DNA Ligase (blue cap)	6 µL
—	**Total volume**	**~84 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 the plate 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

STOPPING POINT You can store PCR products at 10°C overnight. For longer lengths of time, store at –30°C to –10°C.

Purify the unamplified library

IMPORTANT!

- Bring the Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.
- Use freshly prepared 70% ethanol for the next steps. Combine 230 µL of ethanol with 100 µL of Nuclease-free Water per sample.
- Do NOT substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.

1. Carefully remove the plate seal, then add **80 µL (about 1X sample volume) of Agencourt™ AMPure™ XP Reagent** to each library. Pipet up and down 5 times to mix the bead suspension thoroughly with the DNA.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the plate in a DynaMag™–96 Side Magnet (Cat. No. 12331D), then incubate for 5 minutes or until solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.
4. Add **150 µL of freshly prepared 70% ethanol**, move the plate side to side in the magnet to wash the beads, remove, then discard the supernatant. Do not disturb the pellet.
5. Repeat step 4 for a second wash.
6. 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. **Do not overdry**.

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™ Clear 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 the library and dilute

Determine the concentration of each Ion AmpliSeq™ library by qPCR with the Ion Library TaqMan® Quantitation Kit (Cat. No. 4468802) using the steps that follow. Each sample, standard, and negative control is analyzed in duplicate 20-µL reactions.

Note: Exome libraries typically have yields of 100–500 pM.

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 the dilutions 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 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 96-well standard 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). Total reaction volume is 20 µL.
4. Program your real-time instrument:
   - Enter the concentrations of the control library standards.
   - Use ROX™ reference dye as the passive reference dye.
   - Select a reaction volume of 20 µL.
   - You can use the Ion Library qPCR Master Mix on various Thermo Fisher Scientific instruments, as listed in the following table.

Real-time PCR System	Stage	Temperature	Time
7300 Real-Time PCR System	Hold (optional)	50°C	2 minutes
7500 Real-Time PCR System	Hold (polymerase activation)	95°C	2 minutes
7900HT Real-Time PCR system	Cycle (40 cycles)	95°C	15 seconds
7900HT Fast Real-Time PCR System		60°C	1 minute
ViiA™ 7 Real-Time PCR System			
QuantStudio™ 3 or 5 Real-Time PCR System			

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.

   For example:
   - The undiluted library concentration is 300 pM.
   - The library dilution factor is 300 pM/100 pM = 3.
   - Therefore, 1 µL of library that is mixed with 2 µL of Low TE (1:3 dilution) yields ~100 pM.
7. Dilute library to ~100 pM as described, then proceed to combining libraries or template preparation.

(Optional) Combine exome libraries

You can combine two barcoded exome libraries on a single Ion PI™ Chip v3 or Ion 540™ Chip, or four barcoded exome libraries on a single Ion 550™ Chip, for a targeted average coverage depth of approximately 100X.

Note: We recommend a minimum average coverage depth of 100X. Loading fewer libraries per chip yields higher coverage depth. See Appendix B, "Strategies for combining Ion AmpliSeq™ Exome RDY libraries" in the *Ion AmpliSeq™ Exome RDY Library Preparation User Guide* (Pub. No. MAN0010084) for more information.

Store libraries

You can store libraries at 4–8°C for up to 1 month. For longer term, store at –20°C.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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Revision history: Pub. No. MAN0009809

Revision	Date	Description
C.0	17 January 2018	New catalog numbers added for the Ion AmpliSeq™ Exome RDY Kits, and sequencing chips removed from the kits
B.0	19 September 2017	• Updated FuPa digestion and adapter ligation conditions • Added support for IonCode™ Barcode Adapters • Guidance for qPCR program for library quantification aligned with *Ion AmpliSeq™ Library Kit 2.0 User Guide* (Pub No. MAN0006735) • Updated weblinks
A.0	28 March 2014	New Quick Reference