Ion AmpliSeq™ Exome RDY Library Preparation

Using Qubit™ Fluorometer Quantification

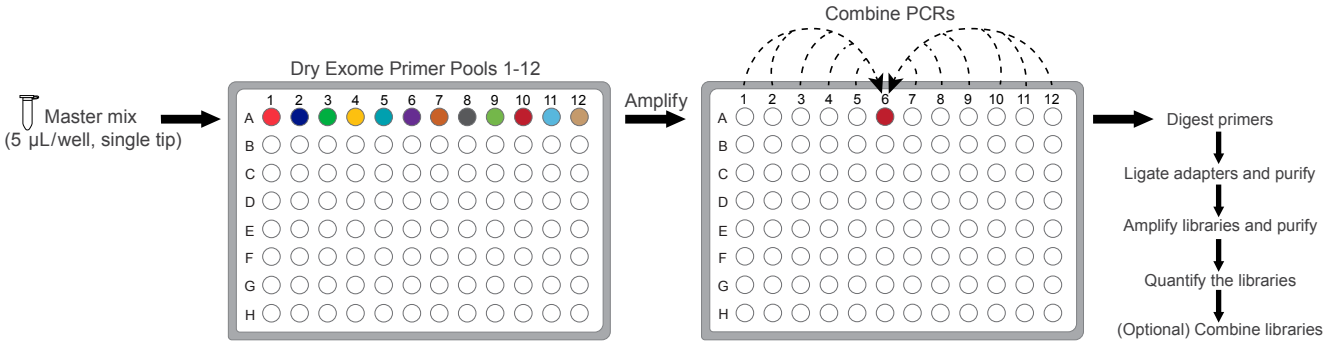
Catalog Numbers A38262, A38264

Pub. No. MAN0009808 **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**.

Amplify the library

1. Remove the plate containing the Ion AmpliSeq™ library from the magnet, then **add 50 µL of 1X Library Amp Mix and 2 µL of 25X Library Amp Primers** to each bead pellet. Pipet the mixture up and down 5 times to mix thoroughly.

2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then 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.

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

Stage	Temperature	Time
Hold	98°C	2 minutes
5 cycles	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold

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

Proceed to "Purify the amplified library".

Purify the amplified library

Perform a two-round purification process with the Agencourt™ AMPure™ XP Reagent:

- **First round at 0.5X bead-to-sample-volume ratio:** High molecular-weight DNA is bound to beads, while amplicons and primers remain in solution. **Save the supernatant.**
- **Second round at 1.2X bead-to-original-sample-volume ratio:** Amplicons are bound to beads, and primers remain in solution. **Save the bead pellet, and elute the amplicons from the beads.**

First-round purification

1. 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 25 µL (0.5X sample volume) of Agencourt™ AMPure™ XP Reagent** (at room temperature) to each plate well containing ~50 µL of sample, then pipet up and down 5 times to mix the bead suspension thoroughly with the DNA.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a DynaMag™–96 Side Magnet for at least 5 minutes or until the solution is clear.
5. Carefully transfer the supernatant from each well to a single well of a new 96-well PCR plate, without disturbing the pellet. Discard the pellet.

Second-round purification

1. To the supernatant from step 5 in the previous procedure, **add 60 µL (1.2X original sample volume) of Agencourt™ AMPure™ XP Reagent**. 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 the magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.
4. **Add 150 µL of freshly prepared 70% ethanol** to each well, then move the plate side to side in the magnet to wash the beads. Remove, then discard the supernatant without disturbing 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.
7. Remove the plate from the magnet, then **add 50 µL of Low TE** to the pellet to disperse the beads. Pipet the mixture up and down 5 times to mix thoroughly.
8. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, and centrifuge to collect droplets. Alternatively, mix by setting a pipettor to 40 µL and pipet the mixture up and down at least 5 times prior to sealing the plate.
9. Incubate at room temperature for at least 2 minutes.
10. Place the plate in the magnet for at least 2 minutes. The supernatant contains the library.

Quantify the library and dilute

Analyze 10 µL of each amplified library using the Qubit™ dsDNA HS Assay Kit (Cat. Nos. Q32851 or Q32854) with the Qubit™ 2.0 Fluorometer, Qubit™ 3.0 Fluorometer, or Qubit™ 4 Fluorometer (Cat. No. Q33226).

Note: Exome libraries typically have yields of 300–3,000 ng/mL.

1. Determine the amplified library concentration:
   a. Make a 1:200 working dilution of Qubit™ dsDNA HS Reagent using the Qubit™ dsDNA HS Buffer. Prepare 200 µL of diluted reagent for each library and standard.
   b. Combine 10 µL of the amplified Ion AmpliSeq™ exome library with 190 µL of diluted Qubit™ dsDNA HS Reagent, mix well, then incubate for at least 2 minutes.
   c. Prepare each Qubit™ dsDNA HS Standard by diluting 10 µL with 190 µL of diluted Qubit™ dsDNA HS Reagent.
   d. Measure the concentration on the Qubit™ 2.0, Qubit™ 3.0, or Qubit™ 4 Fluorometer.
   e. *(Qubit™ 2.0 Fluorometer only)* Calculate the concentration of the undiluted library by multiplying by 20. Alternatively, use the "Calculate Stock Conc." feature on your instrument.
2. Based on the calculated library concentration, determine the dilution that results in a concentration of ~22 ng/mL (which is equivalent to ~100 pM).

   For example:
   - The library concentration is 660 ng/mL.
   - The dilution factor is 660 ng/mL divided by 22 ng/mL = 30.
   - Therefore, 1 µL of library that is mixed with 29 µL of Low TE (1:30 dilution) yields approximately 22 ng/mL.
3. Dilute library to ~22 ng/mL (~100 pM), 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 lengths of time, store at –30°C to –10°C.

Limited product warranty

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Revision history: Pub. No. MAN0009808

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 • Updated for the Qubit™ 4 Fluorometer
B.0	19 September 2017	• Updated FuPa digestion and adapter ligation conditions • Added support for IonCode™ Barcode Adapters • Updated weblinks
A.0	28 March 2014	New Quick Reference

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