# Ion AmpliSeq™ Library Kit 2.0

## DNA Library Preparation with 1- or 2-Pool Panels Using Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ Instrument Quantification

Catalog Numbers 4475345, 4480441, 4480442

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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 Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ Instrument 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<br>Set the cycle number according to the following tables. | Denature | 99°C | 15 seconds |
| | Anneal and extend | 60°C | 4/8/16 minutes<br>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 amplified library using the Qubit™ Fluorometer or Agilent™ 2100 Bioanalyzer™ instrument

Ion AmpliSeq™ libraries must be amplified before quantification to enrich amplifiable material and obtain sufficient material for accurate quantification. Amplify the library using Platinum™ PCR SuperMix High Fidelity, then purify. Quantify the library using a Qubit™ Fluorometer, or the Agilent™ 2100 Bioanalyzer™ instrument. 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.

### Amplify the library

1. Remove the plate with purified libraries from the plate magnet, then add 50 µL of Platinum™ PCR SuperMix High Fidelity and 2 µL of Library Amplification Primer Mix to each bead pellet.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.
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 MicroAmp™ Clear Adhesive Film, place a MicroAmp™ Optical Film Compression Pad on the plate, load in the thermal cycler, then 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 Samples can be stored at –20°C.

### Purify the amplified library

Perform a two-round purification process with the MagMAX™ Pure Bind Beads.

- 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.

**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.

### First-round purification

1. Tap the plate gently on a hard flat surface, or briefly centrifuge to collect the contents, then remove the plate seal.
2. Add 25 µL (0.5X sample volume) of MagMAX™ Pure Bind Beads to each plate well containing ~50 µL of sample. Mix the bead suspension with the DNA thoroughly by pipetting up and down 5 times.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnet, such as the 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 new well of the 96-well PCR plate without disturbing the pellet.

**IMPORTANT!** The supernatant contains the desired amplicons. Do not discard!

### Second-round purification

1. To the supernatant from step 4 in "First-round purification" on page 5, add 60 µL (1.2X original sample volume) of MagMAX™ Pure Bind Beads. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.
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.

**IMPORTANT!** The amplicons are bound to the beads. Save the bead 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 2–5 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.
8. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.
9. Incubate at room temperature for at least 2 minutes.
10. Place the plate in the magnet for at least 2 minutes, then analyze an aliquot of the supernatant as described in:
    - "Qubit™ Fluorometer—Quantify the library and calculate the dilution factor" on page 5 or
    - "Agilent™ 2100 Bioanalyzer™ Instrument—Quantify the library and calculate the dilution factor" on page 5.

**IMPORTANT!** The supernatant contains the desired amplicons. Do not discard!

## Qubit™ Fluorometer—Quantify the library and calculate the dilution factor

1. Determine the amplified library concentration.
    a. Make a 1:200 working dilution of Qubit™ dsDNA HS Reagent using the Qubit™ dsDNA HS Buffer.
    b. Combine 10 µL of the amplified Ion AmpliSeq™ library with 190 µL of dye reagent, mix well, then incubate for at least 2 minutes.
    c. Prepare each Qubit™ standard as directed in the user guide.
    d. Measure the concentration on the Qubit™ 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 ~100 pM.

| Average amplicon size | Concentration in ng/mL (~100 pM) |
|---|---|
| 140 bp | 9 |
| 175 bp | 11 |
| 225 bp | 15 |
| 275 bp | 18 |
| 375 bp | 24 |

3. Dilute library to ~100 pM, combine, then proceed to template preparation, or store libraries as described in "Store libraries" on page 6.

## Agilent™ 2100 Bioanalyzer™ Instrument—Quantify the library and calculate the dilution factor

1. Determine the molar concentration of the amplified library using the Bioanalyzer™ software. Briefly:
    a. Select the **Data** icon in the **Contexts** panel, then view the electropherogram of the sample to be quantified.
    b. Select the **Region Table** tab below, then create a region spanning the desired amplicon peaks. Correct the baseline if needed.

    The molarity is automatically calculated and displayed in the table in pmol/L (pM).

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

3. Dilute the library to ~100 pM, combine, then proceed to template preparation, or store libraries as described in “Store libraries” on page 6.

### *(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. MAN0006943 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.<br>• Added support for MagMAX™ Pure Bind Beads for purification. |
| D.0 | 18 January 2019 | • Support added for Ion 510™ and Ion 550™ Chips.<br>• Support added for Ion GeneStudio™ S5 Systems.<br>• Support added for Ion Torrent™ Dual Barcode Kit 1–96.<br>• 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.<br>• 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). |