iontorrent

Ion AmpliSeq™ HD Library Kit

USER GUIDE

for use with:
Ion AmpliSeq™ HD Dual Barcode Kit 1–24
Ion AmpliSeq™ Made-to-Order Panels

Catalog Numbers A57283, A37694, A53690, A37695

Publication Number MAN0017392

Revision F.0

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

Life Technologies Corporation |
5781 Van Allen Way |
Carlsbad, California 92008 USA

Products manufactured at this site:

- Ion AmpliSeq™ HD Library Kit
- 5X HD Enhancer
- Ion AmpliSeq™ HD Dual Barcode Kit 1–24

Life Technologies Corporation |
6055 Sunol Blvd |
Pleasanton, California 94566 USA

Products manufactured at this site:

- Ion AmpliSeq™ HD Made-to-Order Panels

For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: MAN0017392 F.0 (English)

Revision	Date	Description
F.0	2 January 2024	• Reorganized chapter 1 into two chapters. • Added support for MagMAX™ Pure Bind Beads for purification. • Corrected information about preparing 10X FWD and REV working subpools. Specified primer sizes, concentrations, and dilutions to use in both tube and 384-well plate formats.
E.0	29 March 2023	• Added support for 5X HD Enhancer. • Updated protocol for Ion AmpliSeq™ HD DNA and RNA libraries. • Reorganized content.

The information in this guide is subject to change without notice.

Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product description

The Ion AmpliSeq™ HD Library Kit (Cat. Nos. A57283 and A37694) is used with Ion AmpliSeq™ HD Made-to-Order Panels and the Ion AmpliSeq™ HD Dual Barcode Kit 1–24 (Cat. No. A37695) to generate tagged targeted amplicons libraries for use as part of the Ion Ion AmpliSeq™ HD sequencing workflow on an Ion GeneStudio™ S5 Series Sequencer. Ion AmpliSeq™ HD technology enables scalable polymerase chain reactions (PCR) from 12- to 5000-plex (1 x 5000-plex in a 1-pool panel or 2 x 2500-plex in a 2-pool panel) for Unique Molecular Tags (UMTs) targeted sequencing.

The Ion AmpliSeq™ HD Dual Barcode Kit 1–24 contains primers that are used to prepare dual-barcoded libraries, with barcodes placed at the 5′ end and the 3′ end. The dual-barcode design was specifically developed to reduce the risk of cross-barcode contamination. This configuration supports multiplex sequencing of up to 24 samples on a single chip.

The kit includes the following key features.

- Sample compatibility—The Ion AmpliSeq™ HD Library Kit is compatible with formalin-fixed, paraffin-embedded (FFPE) samples FFPE DNA and FFPE RNA), cell-free TNA (cfDNA and cfRNA), genomic DNA (gDNA), and total RNA.
- Custom design creation—Ion AmpliSeq™ Designer (**AmpliSeq.com**) is used to create custom designs for use with Ion AmpliSeq™ HD technology. The designs are made-to-order and allow the flexibility of creating region designs, hotspot designs, or fusion designs. Changes to the standard protocol can be made to accommodate 3-pool designs. For more information, see "Ion AmpliSeq™ HD Made-to-Order Panels—tube format" on page 11.
- Sensitivity detection—Depending on the sample type, Ion AmpliSeq™ HD technology is capable of levels of detection down to 0.1%. For more information, see Appendix E, "Experimental design of a sequencing experiment using Ion AmpliSeq™ HD technology".
- Multiplexing—The Ion AmpliSeq™ HD Library Kit includes reagents for generating amplicons using Ion AmpliSeq™ HD primers, which are used to prepare libraries for sequencing. The libraries are dual-barcoded at the 5' and 3' ends using the Ion AmpliSeq™ HD Dual Barcode Kit 1–24, which enables the multiplexing of up to 24 samples in a single chip.

- Bi-directional sequencing—The Ion AmpliSeq™ HD Library Kit also provides sequencing information from both strands. This enables better error correction and higher confidence in the quality of your sequence data.
- Library preparation performance—The 5X HD Enhancer, an updated Ion AmpliSeq™ HD library preparation component, improves the product performance by reducing primer-dimer formation during library preparation, and improving amplicon molecular coverage, especially for high-GC amplicons. The 5X HD Enhancer is available bundled with the Ion AmpliSeq™ HD Library Kit (Cat. No. A57283) or separately (Cat. No. A53690).

Ion GeneStudio™ S5 Series instrument reference

In this document, Ion GeneStudio™ S5 Series Sequencer or Ion GeneStudio™ S5 Series System refers generically to the following systems, unless otherwise specified.

- Ion GeneStudio™ S5 System (Cat. No. A38194)
- Ion GeneStudio™ S5 Plus System (Cat. No. A38195)
- Ion GeneStudio™ S5 Prime System (Cat. No. A38196)

Ion AmpliSeq™ HD Made-to-Order Panels with the Ion Torrent™ Genexus™ Integrated Sequencer System

To use Ion AmpliSeq™ HD Made-to-Order Panels with the Ion Torrent™ Genexus™ Integrated Sequencer System, see the *Genexus™ Integrated Sequencer User Guide* (Pub. No. MAN0017910).

Contents and storage

Kit summary

The Ion AmpliSeq™ HD Library Kit is available in two configurations.

- Ion AmpliSeq™ HD Library Kit with HD Enhancer (Cat. No. A57283)—Consists of the Ion AmpliSeq™ HD Library Kit (Cat. No. A37694) bundled with the 5X HD Enhancer (Cat. No. A53690)
- Ion AmpliSeq™ HD Library Kit (Cat. No. A37694)—Stand alone

The Ion AmpliSeq™ HD Library Kit with HD Enhancer and Ion AmpliSeq™ HD Library Kit require the Ion AmpliSeq™ HD Dual Barcode Kit 1–24 and Ion AmpliSeq™ HD Made-to-Order Panels, which must be purchased separately.

5X HD Enhancer (Cat. No. A53690) can also be purchased separately.

Ion AmpliSeq™ HD Library Kit

The Ion AmpliSeq™ HD Library Kit (Cat. No. A37694) provides reagents for manually preparing 24 libraries.

Component	Amount	Storage
4X Ion AmpliSeq™ HD Amplification Mix (purple cap)	216 µL	–30°C to –10°C
Ion AmpliSeq™ HD SUPA Reagent (green cap)	120 µL	
CRC (yellow cap)	220 µL	
Low TE (clear cap)	2 × 1.7 mL	15°C to 30°C[1]

[1] Can be stored at –30°C to –10°C.

5X HD Enhancer

The 5X HD Enhancer (Cat. No. A53690) provides reagents for manually preparing 24 libraries and is required for library preparation.

Component	No. of reactions	Storage
5X HD Enhancer	24	–30°C to –10°C

Ion AmpliSeq™ HD Dual Barcode Kit 1–24

The Ion AmpliSeq™ HD Dual Barcode Kit 1–24 (Cat. No. A37695) provides 24 different barcode primer pairs (barcodes 1–24), and is ordered separately. These barcode primers are required for library preparation when sequencing one or multiple libraries per sequencing chip.

Component	No. of reactions	Storage
Ion AmpliSeq™ HD Dual Barcode Kit 1–24 (96-well plate format, liquid)	Single use reaction per well	–30°C to –10°C

Ion AmpliSeq™ HD Made-to-Order Panels

Ion AmpliSeq™ HD Made-to-Order Panels provide pools of primers for amplification of target regions. Each primer pool consists of 2 subpools (one with forward (FWD) primers and one with reverse (REV) primers). The primers contain proprietary modifications that are required during library preparation for efficient multiplex target assessment.

Visit **AmpliSeq.com** to design and order Ion AmpliSeq™ HD Made-to-Order Panels using Ion AmpliSeq™ Designer. Two configurations are available.

- Tube format—For each panel, the FWD and REV subpools are provided in microcentrifuge tubes. See “Ion AmpliSeq™ HD Made-to-Order Panels—tube format” on page 11.
- 384-well plate format—For each panel, individual primers are provided in each well of a 384-well plate, which must be combined to generate FWD and REV subpools. See “Ion AmpliSeq™ HD Made-to-Order Panels—plate format” on page 10.

Ion AmpliSeq™ HD Made-to-Order Panels—plate format

Note: The 384-well plate format of Ion AmpliSeq™ HD Made-to-Order Panels is available only for DNA panels.

The 384-well plate format of Ion AmpliSeq™ HD Made-to-Order Panels contains all the individual FWD and REV primers in plate wells. You can use the plates to perform the following tasks.

- Remake the entire panel.
- Remake a smaller panel containing a subset of primers.

Each plate well contains one forward (FWD) or reverse (REV) primer in Low TE at a concentration of 307 µM per primer.

To determine the location of the primers in the 384-well plate, go to **AmpliSeq.com**. After creating the designs in Ion AmpliSeq™ Designer, download the results ZIP file, then open the `XYZ_384WellPlateDatasheet.csv` file to view the location of the primers in the plate.

Ion AmpliSeq™ HD Made-to-Order Panels—tube format

Ion AmpliSeq™ HD Made-to-Order Panels in tube format consist of 2 tubes of subpools premixed per pool, one FWD subpool and one REV subpool. The subpools are used directly to generate 10X working FWD and REV subpools.

Table 1 Contents of Ion AmpliSeq™ HD Made-to-Order Panels

Subpool	Volume	Storage[1]
1-pool primer panel (DNA or RNA)[2]		
Pool 1 FWD	6 × 1,500 µL each	–30°C to –10°C
Pool 1 REV	6 × 1,500 µL each	
2-pool primer panel (DNA only)[3]		
Pool 1 FWD	6 × 1,500 µL each	–30°C to –10°C
Pool 1 REV	6 × 1,500 µL each	
Pool 2 FWD	6 × 1,500 µL each	
Pool 2 REV	6 × 1,500 µL each	
3-pool primer panel (DNA only)[4,5]		
Pool 1 FWD	6 × 1,500 µL each	–30°C to –10°C
Pool 1 REV	6 × 1,500 µL each	
Pool 2 FWD	6 × 1,500 µL each	
Pool 2 REV	6 × 1,500 µL each	
Pool 3 FWD	6 × 1,500 µL each	
Pool 3 REV	6 × 1,500 µL each	

[1] Shipped at ambient temperature. Store as indicated.
[2] At least 6 X 500 reactions.
[3] At least 6 X 1000 reactions.
[4] At least 6 X 1500 reactions.
[5] Contact Support if 3-pool primer panel is required.

Workflow

The following workflow includes run planning in Torrent Suite™ Software for sequencing on an Ion GeneStudio™ S5 Series Sequencer. If you are planning to sequence Ion AmpliSeq™ HD libraries on the Genexus™ Integrated Sequencer, see the *Genexus™ Integrated Sequencer User Guide* (MAN0017910) for workflow details.

Design and order Ion AmpliSeq™ HD Made-to-Order Panels

Design and order Ion AmpliSeq™ HD Made-to-Order Panels at AmpliSeq.com

Download and install BED files (page 76)

Download all available BED files specific for your Ion AmpliSeq™ HD panels from AmpliSeq.com, or contact your field service representative if you need help accessing the BED files.

From library preparation to sequencing results report

Create a custom workflow in Ion Reporter™ Software (page 77)

Contact support to activate Ion AmpliSeq™ HD analysis workflow templates in Ion Reporter™ Software to create a custom analysis workflow for use with Ion AmpliSeq™ HD panels.

Create a custom Planned Run template in Torrent Suite™ Software (page 22)

Using a preinstalled Ion AmpliSeq™ HD Planned Run template, set up a customized Planned Run template for reuse when the same conditions are used for multiple runs.

Create an assay-specific Planned Run in Torrent Suite™ Software (page 25)

Use a customized Planned Run template to create a Planned Run.

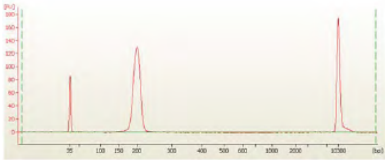
Prepare Ion AmpliSeq™ HD DNA libraries (page 32)

OR

Prepare Ion AmpliSeq™ HD RNA libraries (page 49)

Amplify the targets, partially digest the amplicons, amplify the libraries with barcoded primers, then purify.

Quantify libraries with the Agilent™ 2100 Bioanalyzer™ Instrument and calculate the dilution factor (page 59)



Combine libraries (page 62)

To sequence multiple barcoded libraries on a single chip, combine libraries before template preparation.

From library preparation to sequencing results report

Proceed to prepare template, then sequence

Perform template preparation and sequencing as described in the instrument user guide. For details on compatible chips, kits, and instruments, see page 61.

Review plugin results and visualize variants

- Use the Ion Torrent™ coverageAnalysis plugin or the molecular CoverageAnalysis plugin to view statistics and graphs that describe the level of sequence coverage produced for targeted genomic regions.

  For information about setting up and performing data analysis with Torrent Suite™ Software, see the Torrent Suite™ Software help system or the *Torrent Suite™ Software 5.18 User Guide* (Pub. No. MAN0026163).

- Ion Reporter™ Software analyses are performed automatically when the data files are uploaded from Torrent Suite™ Software.

  For information about using the suite of bioinformatics tools in Ion Reporter™ Software to streamline variant analysis and reporting, see the Ion Reporter™ Software help system or the *Ion Reporter™ Software 5.20 User Guide* (Pub No. MAN0028322).

2 Reagents, supplies, and required materials

This chapter lists the reagents, supplies, and materials needed to operate the Ion GeneStudio™ S5 Series Sequencer, and provides consumables ordering and storage information. Reagents and supplies can be ordered as kits and starter packs, but most consumables can also be ordered individually as your needs require.

Note: Consumables that have catalog numbers are orderable. Components that have part numbers cannot be ordered individually.

Required materials not supplied

In addition to a library kit, library barcodes, and panel, you need the following materials and equipment. Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
One of the following thermal cyclers. • *(Recommended)* Veriti™ Dx 96-well Thermal Cycler, 0.2 mL • VeritiPro™ Thermal Cycler, 96-well • ProFlex™ 96-well PCR System • SimpliAmp™ Thermal Cycler, 96-well • Veriti™ 96-Well Thermal Cycler[1]	 • 4452300 • A48141 • 4484075 • A24811 • 4375786
One of the following kits. • *(If quantifying using real-time PCR)* Ion Library TaqMan™ Quantitation Kit • Agilent™ High Sensitivity DNA Kit and Agilent™ 2100 Bioanalyzer™ Instrument • Ion Library Equalizer™ Kit and Platinum™ PCR SuperMix High Fidelity	 • 4468802 • 5067-4626, G2939BA (Agilent) • 4482298 and 12532016

(continued)

Item	Source
One of the following plates. • *(Recommended)* MicroAmp™ EnduraPlate™ Optical 96-Well Clear Reaction Plates with Barcode • MicroAmp™ Optical 96-Well Reaction Plate with Barcode	 • 4483354 • 4306737
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical Film Compression Pad	4312639
Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5-mL	13-698-791
Fisher Scientific™ Mini Plate Spinner Centrifuge, or equivalent 96-well plate centrifuge	14-100-143
One of the following purification reagents. • MagMAX™ Pure Bind Beads (preferred) • Agencourt™ AMPure™ XP Reagent (optional)	 • A58521, A58522, A58523 • NC9959336, NC9933872
DynaMag™–96 Side Magnet, or other plate magnet	12331D
(FFPE DNA only) Uracil-DNA Glycosylase, heat-labile	78310100UN
Nuclease-Free Water (not DEPC-Treated)	AM9932
Ethanol, Absolute, Molecular Biology Grade	BP2818500
Pipettors, 2–200 µL, and low-retention filtered pipette tips	**MLS**
(RNA only) Ion Torrent™ NGS Reverse Transcription Kit	A45003
(384-plate format only) 15-mL and 50-mL centrifuge tubes	**MLS**

[1] Supported but no longer available for purchase.

Recommended materials and equipment

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Item	Source
Additional equipment	
One of the following Applied Biosystems™ real-time PCR instruments. • 7500 Real-Time PCR System • 7900HT Fast Real-Time PCR System[1] • StepOne™ Real-Time PCR System • StepOnePlus™ Real-Time PCR System • ViiA™ 7 Real-Time PCR System • QuantStudio™ Real-Time PCR Systems	**MLS**
Light Labs 96-WELL ALUMINUM BLOCK	NC9820161
MicroAmp™ Adhesive Film Applicator	4333183
Nucleic acid isolation	
MagMAX™ DNA Multi-Sample Ultra 2.0 Kit	A36570
PureLink™ Genomic DNA Mini Kit	K1820-01
MagMAX™ Cell-Free DNA Isolation Kit	A29319
MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit	A36716
RecoverAll™ Multi-Sample RNA/DNA Workflow	A26069
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975
MagMAX™ FFPE DNA/RNA Ultra Kit	A31881
MagMAX™ *mir*Vana™ Total RNA Isolation Kit	A27828
PureLink™ Total RNA Blood Kit	K156001
Nucleic acid quantification	
Qubit™ 4 Fluorometer[2] with one of these quantitation kits. • *(DNA)* Qubit™ dsDNA HS Assay Kit • *(RNA)* Qubit™ RNA HS Assay Kit	Q33238 Q32851, Q32854 Q32852, Q32855
Controls	
Ion S5™ Controls Kit Plus	A30729
Seraseq™ Fusion RNA Mix v4	0710-0497 (**seracare.com**)

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Item	Source
Seraseq™ FFPE Tumor Fusion RNA v4 Reference Material	0710-0496 (**seracare.com**)
AcroMetrix™ Oncology Hotspot Control *(FFPE DNA)*	969056
Horizon™ Multiplex I cfDNA Reference Standard Set	HD780 (**horizondiscovery.com**)

[1] Supported but no longer available for purchase.
[2] The Qubit™ 2.0 Fluorometer & Qubit™ 3.0 Fluorometer are supported but no longer available for purchase.

3 Before you begin

The guidelines presented in this chapter are for library preparation using the 5X HD Enhancer. If you are using CRC, see Appendix C, "Prepare Ion AmpliSeq™ HD DNA libraries using CRC" or Appendix D, "Prepare Ion AmpliSeq™ HD RNA libraries without using 5X HD Enhancer".

Procedural guidelines

IMPORTANT! Do not combine FWD and REV primer subpools for storage. Primer subpools must remain separate until they are combined during the reaction setup.

- Minimize freeze-thaw cycles of Ion AmpliSeq™ HD panels by aliquoting primer subpools for your experiments. Panels can be stored 4°C for 1 month. For longer term, store at –20°C.
- Use a calibrated thermal cycler that is specified in "Required materials not supplied" on page 15.
- Pipet viscous solutions, such as 4X Ion AmpliSeq™ HD Amplification Mix, Ion AmpliSeq™ HD SUPA Reagent, and HD Enhancer slowly. After thawing the capped tubes, vortex for 10 seconds to ensure complete mixing.
- Arrange samples in alternating columns on the plate for easier pipetting with multichannel pipettes during purification with the DynaMag™–96 Side Magnet or other plate magnet.
- Prepare fresh 70% ethanol each day.
- Use the default ramping rate when entering the cycling program for the following thermal cyclers.
  - Veriti™ Dx 96-well Thermal Cycler
  - VeritiPro™ Thermal Cycler, 96-well
  - SimpliAmp™ Thermal Cycler
  - ProFlex™ Thermal Cycler
  - Veriti™ 96-Well Thermal Cycler (Discontinued. Supported but no longer available for purchase. Use VeritiPro™ Thermal Cycler, 96-well (Cat. No. A48141) or Veriti™ Dx 96-well Thermal Cycler (Cat. No. 4452300).

Tips

- Target amplification reaction master mixes can be made with 4X Ion AmpliSeq™ HD Amplification Mix, 5X HD Enhancer, and primer pools, transferred to a 96-well plate, then sample DNA added. To avoid pool imbalance, ensure that you are adding equal amounts of DNA to each pool of 2-pool and 3-pool panel reactions.
- Arrange samples in columns on the plate for easier pipetting with multichannel pipettes during purification with the DynaMag™–96 Side Magnet.
- If you observe evaporation in target amplification reactions, avoid using outside wells.
- Plate seals can be firmly applied using the MicroAmp™ Adhesive Film Applicator. Plate seals can be removed with less effort when hot. Try removing seals right after taking the plate out of the thermal cycler, then briefly centrifuge.
- When using the Agilent™ 2100 Bioanalyzer™ Instrument, for libraries with low yield (below 0.5 nM) and no detectable product on the bioanalyser, it may not be worth trying to further quantify for sequencing. There may be a problem in either the sample or the library preparation, or both. If library yield is below 100 pM, libraries can still be sequenced by adding a proportionally larger volume to a combined library or template preparation.
- When transfer to a new plate is specified, solutions can be transferred to a clean well in the same plate instead, if desired.

Guidelines for preventing contamination

- Use good laboratory practices to minimize cross-contamination of products. If possible, perform PCR setup in an area or room that is free of amplicon contamination. Always change pipette tips between samples.
- When preparing libraries, we recommend that you alternate barcodes between consecutive sequencing runs to prevent carryover contamination. The following table provides an example.

Library type	Barcode usage		
	System run 1	System run 2	System run 3
Positive control	1	9	17
Sample	2	10	18
Sample	3	11	19
Sample	4	12	20
Sample	5	13	21
Sample	6	14	22
Sample	7	15	23
No-template control (NTC)	8	16	24

4 Create a Planned Run in Torrent Suite™ Software

IMPORTANT! This kit is compatible with Torrent Suite™ Software 5.10 or later and Ion Reporter™ Software 5.10 or later. Torrent Suite™ Software 5.10 or later is required to access the preinstalled Planned Run templates and to enable the data analysis and reporting options that are described in this chapter. Before proceeding, check for updates to the Torrent Suite™ Software, Ion Reporter™ Software, and Ion Chef™ System software, then install available updates. Contact your service representative for help with upgrading the software.

Torrent Suite™ Software Planned Run templates for use with Ion AmpliSeq™ HD panels

Planned Runs contain all the settings that are used in a sequencing run, including number of flows, kit types, barcodes, sample information, and reference files (if any). Planned Runs are used to track samples, chips, and reagents throughout the sequencing workflow, from template preparation on the Ion Chef™ Instrument through sequencing on the Ion GeneStudio™ S5 Series System and subsequent data analysis. Each chip that is prepared in an Ion Chef™ run requires its own Planned Run.

The Torrent Suite™ Software 5.10 or later contains 8 preinstalled Planned Run templates for use with Ion AmpliSeq™ HD panels.

Research application	Torrent Suite™ Software template name	Description
Ion AmpliSeq™ HD	Ion AmpliSeq™ HD for Liquid Biopsy - DNA	DNA-only Planned Run template for liquid biopsy samples
	Ion AmpliSeq™ HD for Liquid Biopsy - Fusions	Fusions-only Planned Run template for liquid biopsy samples
	Ion AmpliSeq™ HD for Liquid Biopsy - DNA and Fusions - Single Library	DNA and Fusions Planned Run template for single library liquid biopsy samples; use this template if the DNA and RNA libraries were prepared from a single sample and have the same barcode.

(continued)

Research application	Torrent Suite™ Software template name	Description
Ion AmpliSeq™ HD	Ion AmpliSeq™ HD for Liquid Biopsy - DNA and Fusions - Separate Libraries	DNA and Fusions Planned Run template for liquid biopsy samples with separate libraries; use this template if the DNA and RNA libraries were prepared separately and each have their own barcode.
	Ion AmpliSeq™ HD for Tumor - DNA	DNA-only Planned Run template for tumor samples
	Ion AmpliSeq™ HD for Tumor - Fusions	Fusions-only Planned Run template for tumor samples
	Ion AmpliSeq™ HD for Tumor - DNA and Fusions - Single Library	DNA and Fusions Planned Run template for single library tumor samples; use this template if the DNA and RNA libraries were prepared from a single sample and have the same barcode.
	Ion AmpliSeq™ HD for Tumor - DNA and Fusions - Separate Libraries	DNA and Fusions Planned Run template for tumor samples with separate libraries; use this template if the DNA and RNA libraries were prepared separately and each have their own barcode.

Create a custom Planned Run template

We recommend that you set up a customized Planned Run template for reuse when the same conditions are used for multiple runs.

1. Sign in to Torrent Suite™ Software.
2. In the **Plan** tab, in the **Templates** screen, click **AmpliSeq HD** in the left navigation menu.
3. In the **AmpliSeq HD** table, find the system template that best fits your research application, then click **Settings** ▸ **Copy** in the row of the template.
   The **Copy Template** workflow bar opens to the **Save** step.

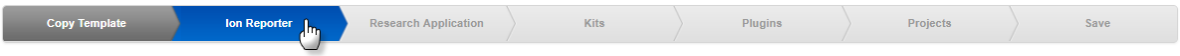
4. In the **Save** step, complete the required information.

Item	Action
Template Name	Enter a name for your custom Planned Run template.
Set as Favorite	Select this checkbox to add your custom template to the **Favorites** list.
Analysis Parameters	Select **Default** to accept default analysis parameter settings (recommended). Advanced users can select **Custom** to customize analysis parameters and edit appropriate analysis fields. For more information, see the Torrent Suite™ Software help system or the *Torrent Suite™ Software 5.18 User Guide* (Pub. No. MAN0026163) .
DNA Reference Library	Select the reference library file that is appropriate for your sample. Depending on your application, you may have to select separate DNA, RNA, and Fusions reference library files.
DNA Target Regions[1]	Select the Target Regions BED file that is appropriate for your sample. Depending on your application, you may have to select separate DNA and Fusions Target Regions files.
DNA Hotspot Regions[1]	Select the hotspot (BED or VCF) file that is appropriate for your sample.

[1] Check with your service representative for updates to ensure the most current files are being used. For BED file installation instructions, see “Download and install BED files” on page 76.

As you make your selections, your settings are updated in the **Summary** pane.

5. In the **Copy Template** workflow bar, click the **Ion Reporter** step, then ensure that the **Sample Grouping** selection matches your research application.



6. Set up the transfer of the completed run results to a specified Ion Reporter™ Server.

IMPORTANT! Before you set up automatic transfer of sequencing data to Ion Reporter™ Software, you must configure your Ion Reporter™ Software account and create a custom Ion Reporter™ Software workflow that is specific for your Ion AmpliSeq™ HD panels. For more information about configuring your Ion Reporter™ Software, see the Torrent Suite™ Software help system or the *Torrent Suite™ Software 5.18 User Guide* (Pub. No. MAN0026163). To create a custom Ion Reporter™ Software workflow for use with Ion AmpliSeq™ HD panels, see “Create a custom workflow in Ion Reporter™ Software” on page 77.

From the **Existing Workflow** list, select the server name and select your custom Ion Reporter™ Software workflow.

7. Click **Next**.

8. In the **Research Application** step, ensure that the **Research Application** and **Target Technique** selections are correct, then click **Next**.

9. In the **Kits** step, select the required information.

Item	Selection		
Instrument	Ion GeneStudio™ S5 Series		
Library Kit Type	Ion AmpliSeq™ HD Library Kit		
Sample Preparation Kit	*(Optional)* Select the sample preparation kit that is used to prepare samples.		
Chip Type	Ion 530™ Chip	Ion 540™ Chip	Ion 550™ Chip[1]
Template Kit Ensure that **IonChef** is selected.	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef	Ion 540™ Kit – Chef	Ion 550™ Kit – Chef
Sequencing Kit	Ion S5™ Sequencing Kit		
Control sequence	*(Optional)* Select a control sequence.		
Barcode Set	Ion AmpliSeq™ HD Dual Barcode Kit 1–24		
Flows	550		

[1] The Ion 550™ Chip is compatible only with the Ion GeneStudio™ S5 Plus and Ion GeneStudio™ S5 Prime model sequencers.

10. In the **Advanced Settings** section of the **Kits** step, select one of the following actions, then click **Next**.
    - If you are using the Ion 530™ Chip or Ion 540™ Chip, ensure that **Use Recommended Defaults** is selected.
    - If you are using the Ion 550™ Chip, for Torrent Suite™ Software 5.18 and later, select **Customize**, then select **MRD 550 Templating Script** for **Templating Protocol**.
    - If you are using the Ion 550™ Chip, for Torrent Suite™ Software 5.16 and earlier, ensure that **Use Recommended Defaults** is selected.
11. Select or edit the optional information fields that apply to your sequencing run, then click **Next**.
12. In the **Plugins** step, verify that the **coverageAnalysis** and **molecularCoverageAnalysis** plugins are selected.
13. Click **Next**.
14. *(Optional)* In the **Projects** step, select a project to receive data from runs planned in this template, then click **Next**.

    For more information about **Projects**, see the Torrent Suite™ Software help system or the *Torrent Suite™ Software 5.18 User Guide* (Pub. No. MAN0026163).
15. In the **Save** step, enter a template name in **Template Name (required)**, then click **Copy Template** to save your custom Planned Run template.

The customized template is now available in the **Templates** screen under the **AmpliSeq HD** research application category.

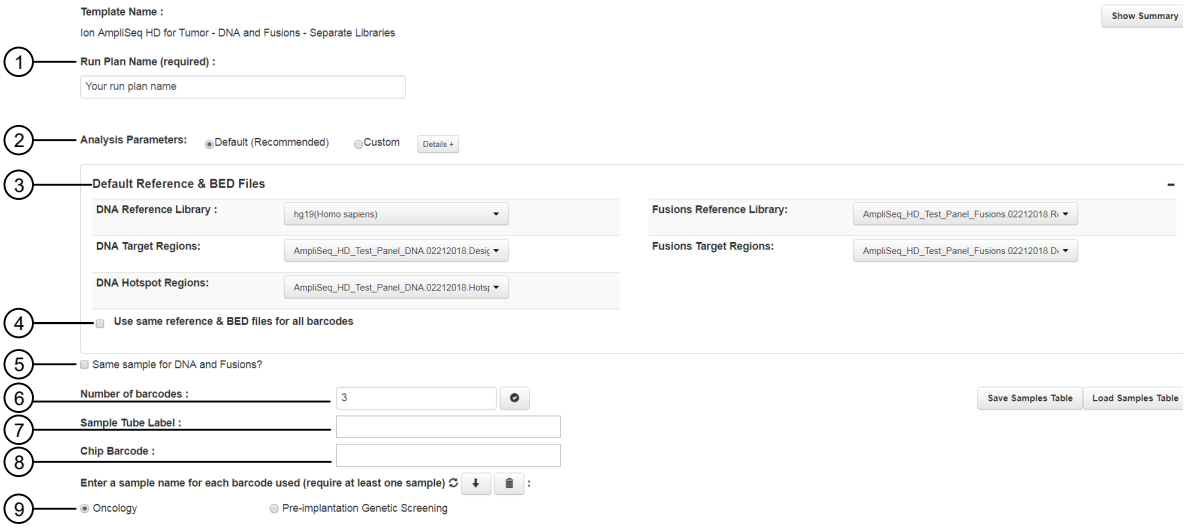
Create a Planned Run

To create a Planned Run, we recommend that you use a Planned Run template that is customized for use with Ion AmpliSeq™ HD libraries. To create a custom Planned Run template, see "Create a custom Planned Run template" on page 22.

1. Sign in to Torrent Suite™ Software for the Ion Torrent™ Server that is connected to your Ion Chef™ System.
2. In the **Plan** tab, in the **Templates** screen, click **AmpliSeq HD** in the left navigation menu.
3. In the **Template Name** column, click your customized Planned Run template name.
   The **Create Plan** workflow bar opens to the **Plan** step.
4. Complete the **Plan** step.

Callout	Item	Action
1	**Run Plan Name**	Enter a Run Plan name.
2	**Analysis Parameters**	Ensure that **Default (Recommended)** is selected.
3	**Default Reference & BED Files**	Select the default reference sequence file and BED files for your sequencing run.
4	**Use same reference & BED files for all barcodes**	Deselect if assigning different files to individual samples.
5	**Same sample for DNA and Fusions?**	Deselect if using different samples for DNA and Fusions.
6	**Number of barcodes**	Enter the number of barcodes to be used in this run, then click ✔.
7	**Sample Tube Label**	Enter or scan the barcode of the Ion Chef™ sample tube to be used in the run.
8	**Chip Barcode**	*(Optional)* Enter or scan the chip barcode.
9	**Oncology**[1]	Select **Oncology**, if applicable.
	Pre-implantation Genetic Screening[1]	Select the **Pre-implantation Genetic Screening**, if applicable.

[1] The **Oncology** option is not available until an Ion Reporter™ Software account has been configured and selected.



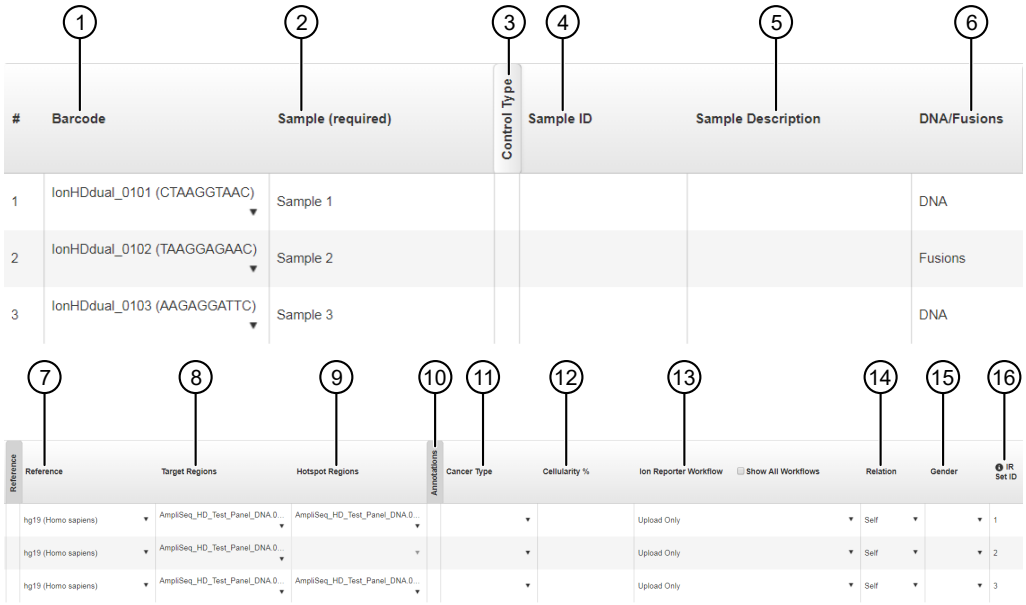
5. Enter sample information.

Callout	Item	Action
1	**Barcode**	For each sample, select the barcode that is used.
2	**Sample (required)**	Click ⬇ above the table to auto-generate sample names or click the field and enter a unique name for each sample in the **Sample (required)** column of the table. **IMPORTANT!** Remove any spaces from the sample names. We recommend that sample names (either auto-generated or user-defined) are unique for every sequencing run.
3	**Control Type**[1]	Select **No Template Control** to designate a sample as a no template control.
4	**Sample ID**	*(Optional)* Click the field, then enter a sample ID.
5	**Sample Description**	*(Optional)* Click the field, then enter a sample description.
6	**DNA/Fusions**	*(DNA and Fusions - Separate Libraries templates only)* Select **DNA** or **Fusions** to denote each sample.
7	**Reference**[1]	*(Optional)* If using different reference sequence files for each sample, select a reference, where applicable.
8	**Target Regions**	If using different Target Regions files for each sample, select a reference, where applicable.
9	**Hotspot Regions**	If using different Hotspot Regions files for each sample, select a reference, where applicable.
10	**Annotations**[1]	Click to reveal **Cancer Type** and **Cellularity %** columns if you selected **Oncology** above the table. If you selected **Pre-implantation Genetic Screening**, then **Biopsy Days**, **Couple ID**, and **Embryo ID** columns appear instead.
11	**Cancer Type**	*(Optional)* Click the field, then select from the dropdown list. Click ⬇ to copy the entry to all the rows.

(continued)

Callout	Item	Action
12	**Cellularity %**	*(Optional)* Click the field, then enter a value. Click to copy the entry to all the rows.
13	**Ion Reporter Workflow**	*(Optional)* Ensure that the correct workflow is selected, if applicable.
14	**Relation**	*(Optional)* Click the field, then ensure the correct value is auto-populated. Select from the dropdown list to change.
15	**Gender**	*(Optional)* Click the field, then select from the list. Click to copy the entry to all rows.
16	**IR Set ID**	*(Optional)* **IR Set ID** links individual samples for analysis. Ensure that the auto-populated value is unique to each sample. Select from the list to change.

[1] Click vertical column headings to reveal additional columns.



6. Click **Plan Run**.

The run is listed in the **Planned Runs** screen under the name that you specified and is used by the Ion Chef™ System when the associated Ion Chef™ Library Sample Tube is loaded on the instrument.

5 Prepare 10X FWD and REV working subpools

IMPORTANT! For RNA panels in tube format, proceed to library preparation (Chapter 7, "Prepare Ion AmpliSeq™ HD RNA libraries"). The stock RNA panel subpools are the 10X RNA working FWD and REV subpools.

Prepare 10X DNA FWD and REV working subpools—tube format

If you have already prepared the 10X DNA FWD and REV working subpools, proceed directly to Chapter 6, "Prepare Ion AmpliSeq™ HD DNA libraries".

Note: If you are preparing 10X working FWD and REV subpools from 384-well plate format panels, see "Prepare 10X DNA FWD and REV working subpools—384-well plate format" on page 29.

IMPORTANT!

- Keep all reagents on ice or in a prechilled 4°C cold block during preparation.
- Do not combine DNA panel FWD and REV primer subpools for storage. Primer subpools must remain separate until combined in the target amplification reaction.
- For each DNA panel pool, prepare 10X working panel subpools for both the forward primer (FWD) and reverse primer (REV).
- For RNA panels, use the RNA stock subpools without dilution. The FWD and REV stock RNA subpools are the 10X RNA working FWD and REV subpools. Proceed to Chapter 7, "Prepare Ion AmpliSeq™ HD RNA libraries".

1. For each DNA panel pool, thaw the DNA stock panel FWD and REV subpools on ice.
2. Before proceeding, ensure that all subpools are completely thawed with no ice remaining, briefly vortex, then centrifuge briefly to collect contents.
3. Determine the number of primers in the FWD and REV subpools for each panel pool.

   The number of amplicons per pool is found in the `designed.bed` file or the panel page on **AmpliSeq.com**. The number of primers in each subpool is equal to the number of amplicons in the corresponding pool.

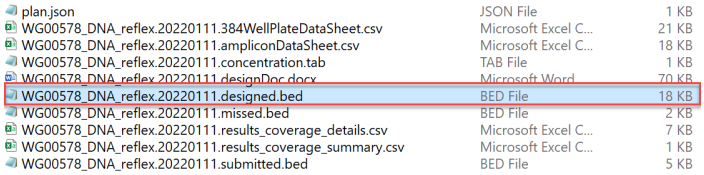


Figure 1 `designed.bed` **file**

4. For FWD and REV subpools with 1229–5000 primers, calculate the stock panel FWD and REV subpool primer concentration and dilution factor.

   Concentration = (307 / number of primers) x 1000

   Dilution factor for panels with 1229–2000 primers = concentration / 100

   Dilution factor for panels with 2001–4000 primers = concentration / 60

   Dilution factor for panels with 4001–5000 primers = concentration / 30

5. Dilute each DNA stock panel FWD and REV subpools with Low TE to create 10X working subpools.

Number of primers in FWD or REV subpools	Stock panel FWD and REV subpool concentration (nM)	10X working subpool primer concentration (nM)	Dilution
12–399	500	100	1:5
400–1,228	250	100	1:2.5
1,229–2,000	Variable[1]	100	1: dilution factor[1]
2,001–4,000	Variable[1]	60	1: dilution factor[1]
4,001–5,000	Variable[1]	30	1: dilution factor[1]

[1] Determined in step 4.

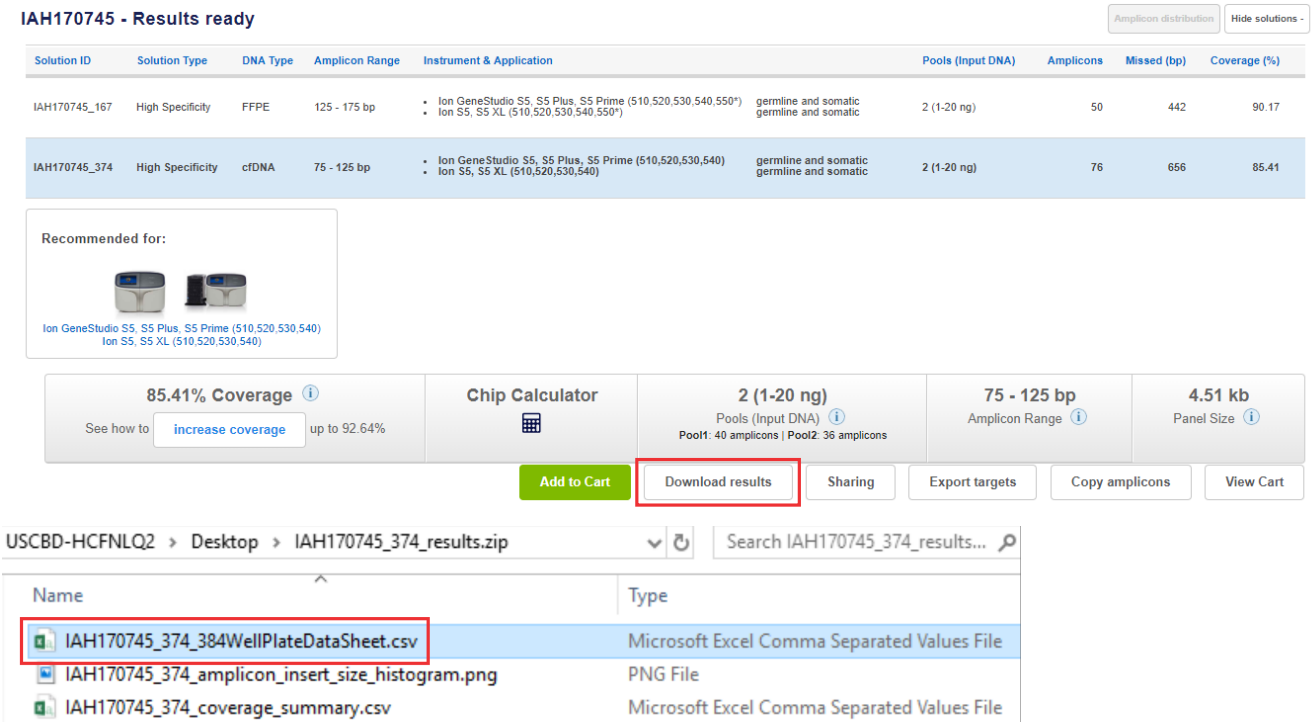
STOPPING POINT 10X DNA working panel FWD and REV subpools can be stored at 4°C for one week. For longer term storage, aliquot and store subpools at -20°C.

Prepare 10X DNA FWD and REV working subpools—384-well plate format

The 384-well plate configuration of Ion AmpliSeq™ HD Made-to-Order DNA Panels contains all the individual FWD and REV primers in plate wells. You can use the plates to perform the following tasks.

- Remake the entire panel.
- Remake a smaller panel containing a subset of primers.

To determine the location of the primers in the 384-well plate, go to **AmpliSeq.com**. After you create the designs in Ion AmpliSeq™ Designer, download the results ZIP file, then open the `XYZ_384WellPlateDatasheet.csv` file to view the location of the primers in the plate.



IMPORTANT!

- Keep all reagents on ice or in a pre-chilled 4°C cold block during reaction setup.
- Do not combine DNA panel FWD and REV primer subpools for storage. Primer subpools must remain separate until combined in the target amplification reaction.
- For each DNA panel pool, prepare 10X working panel subpools for both the forward primer (FWD) and reverse primer (REV).

1. Thaw the Ion AmpliSeq™ HD sealed primer plate on ice.

2. Ensure that the sealed primer plate has completely thawed with no visible ice present, vortex to mix, then centrifuge briefly to collect contents.

3. Carefully remove the plate seal, for each FWD and REV subpool, combine 2 µL of each primer into a 15-mL conical tube (or a 50-mL conical tube for subpools with more than 4,000 primers), then add Low TE (10 mM Tris, pH 8.0; 0.1 mM EDTA) to the final volume in the Table 2.

   IMPORTANT!

   - For each panel pool, prepare both the 10X working panel FWD and REV subpools. 1-pool panels require 2 subpools (FWD and REV), 2-pool panels require 4 subpools (one FWD and REV for each pool), and 3-pool panels require 6 subpools (one FWD and REV for each pool).
   - Only combine primers for each subpools. Do not mix FWD and REV primers. The DNA 10X working panel FWD subpool must contain only FWD primers and DNA 10X working panel REV subpool must contain only REV primers.

Each plate well contains the forward (FWD) or reverse (REV) primer in Low TE at a concentration of 307 µM per primer.

Table 2 Concentration of DNA 10X working FWD and REV subpools

Number of primers in FWD or REV subpools	Final concentration of 10X working panel FWD and REV subpools (nM)	Final volume of 10X working panel FWD and REV subpools (2-µL volume for each primer)
12–2,000 primers	100	6.140 mL
2,001–4,000 primers	60	10.233 mL
4,001–5,000 primers	30	20.466 mL

STOPPING POINT 10X FWD and REV working subpools can be stored at 4°C for one week. For longer term storage, aliquot and store subpools at -20°C.

6 Prepare Ion AmpliSeq™ HD DNA libraries

Guidelines for DNA isolation, quantification, and input

- For recommended isolation kits, see “Recommended materials and equipment” on page 17.
- cfDNA, cfTNA, FFPE DNA, and gDNA are compatible.
- We recommend that you use the Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) to quantify DNA samples.
- Quantification methods such as densitometry (for example, using a NanoDrop™ Spectrophotometer) are not recommended because they are not specific for DNA. Use of these methods can lead to gross overestimation of the sample DNA concentration, under-seeding of the target amplification reaction, and low library yields.
- Library yield is not indicative of sequencing performance.
- In general, library yield from high quality DNA is greater than that from degraded samples (for example, FFPE).
- If the library yield is below 100 pM, use a proportionally larger volume of the library during template preparation or when combining libraries.

Table 3 Minimum concentration requirements for input DNA to achieve 0.1% limit of detection (LOD)

Sample	1 primer pool	2 primer pools
cfDNA, cfTNA, and gDNA	≥1.9 ng/µL	≥3.8 ng/µL
FFPE DNA	≥2.1 ng/µL	≥4.7 ng/µL

For more information, see Appendix E, "Experimental design of a sequencing experiment using Ion AmpliSeq™ HD technology".

Before each use of the kit

- Thaw the 4X Ion AmpliSeq™ HD Amplification Mix, 5X HD Enhancer, Ion AmpliSeq™ HD SUPA Reagent, and Ion AmpliSeq™ HD Dual Barcode Kit 1–24 on ice. Keep on ice during the procedure.
- Ensure that you have prepared 10X Working Panel FWD and REV subpools. See "Prepare 10X DNA FWD and REV working subpools—tube format" on page 28 or "Prepare 10X DNA FWD and REV working subpools—384-well plate format" on page 29.

Remove deaminated bases from FFPE DNA

IMPORTANT! If using DNA isolated from sources other than FFPE tissue, proceed directly to "Set up DNA molecular tagging reactions" on page 34.

FFPE preservation methods can lead to significant cytosine deamination of the isolated DNA, resulting in decreased sequencing quality. When using the Ion AmpliSeq™ HD Library Kit, deaminated cytosine (uracil) bases should be enzymatically removed by treatment with Uracil DNA Glycosylase (UDG) before the target amplification reaction.

1. For each FFPE DNA sample, add the following components to a single well of a 96-well PCR plate.
   - For optimal results, to achieve 0.1% LOD, use 50 ng of input DNA.
   - If the amount of input DNA is less than 50 ng, use the maximum amount available. The minimum input per sample is 20 ng.
   - For 2-pool primer panels, prepare 2 wells, one for each sub pool. Each well requires 20–60 ng.
   - For 3-pool primer panels, prepare 3 wells, one for each pool. Each well requires 20–60 ng.

Component	Volume		
	1-pool primer panel	2-pool primer panel	3-pool primer panel
20–60 ng FFPE DNA	≤9.5 µL	≤4.25 µL	≤2.5 µL
UDG, heat-labile	1 µL	1 µL	1 µL
Low TE	to 10.5 µL	to 5.25 µL	to 3.5 µL
Total volume	**10.5 µL**	**5.25 µL**	**3.5 µL**

2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.
   - Alternatively, mix the reaction by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film.
   - To prevent evaporation during UDG treatment, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

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

Temperature	Time
37°C	2 minutes
60°C	10 minutes
4°C	Hold (≤1 hour)

4. Remove the plate from the thermal cycler, then centrifuge briefly to collect the contents.

STOPPING POINT Reactions can be stored at –20°C long term.

Set up DNA molecular tagging reactions

Prepare molecular tagging reactions—1-pool primer panel

IMPORTANT!

- Keep all reagents on ice or in a prechilled 4°C cold block during reaction setup.
- Do not combine 10X DNA working panel FWD and REV primer subpools for storage. DNA working panel FWD and REV primer subpools must remain separate until combined in step 6.
- 4X Ion AmpliSeq™ HD Amplification Mix and 5X HD Enhancer are viscous. Pipet slowly and mix thoroughly.
- For legacy protocols without using 5X HD Enhancer, see Appendix C, “Prepare Ion AmpliSeq™ HD DNA libraries using CRC”.
- Treat FFPE DNA samples with UDG before use. See “Remove deaminated bases from FFPE DNA” on page 33.

IMPORTANT! Before performing the molecular tagging reaction, prepare 10X DNA working panel FWD and REV primer subpools. See “Prepare 10X DNA FWD and REV working subpools—tube format” on page 28 or “Prepare 10X DNA FWD and REV working subpools—384-well plate format” on page 29.

1. Thaw the following components on ice for at least 10 minutes before use.
   - DNA samples
   - 4X Ion AmpliSeq™ HD Amplification Mix
   - 5X HD Enhancer
   - 10X DNA working panel FWD and REV primer subpools (prepared in “Prepare 10X DNA FWD and REV working subpools—tube format” on page 28)
2. Incubate the 4X Ion AmpliSeq™ HD Amplification Mix at room temperature for 10 minutes.

3. Chill a 96-well plate on ice.
   - For FFPE DNA samples, if the treated samples were frozen, thaw the sealed plate with the frozen pretreated samples, centrifuge briefly to collect the contents, carefully remove the plate seal, then place the plate on ice or in a prechilled 4°C cold block.
   - For non-FFPE DNA samples, place a 96-well PCR plate on ice.

4. Ensure that the DNA samples, 5X HD Enhancer, and Panel FWD and REV primer subpools are completely thawed, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.

5. Ensure that the 4X Ion AmpliSeq™ HD Amplification Mix is completely thawed and that there is no visible precipitate, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents. If you see precipitate, incubate 4X Ion AmpliSeq™ HD Amplification Mix the at room temperature for 10–20 minutes, vortex to completely dissolve the precipitate, then centrifuge briefly to collect the contents.

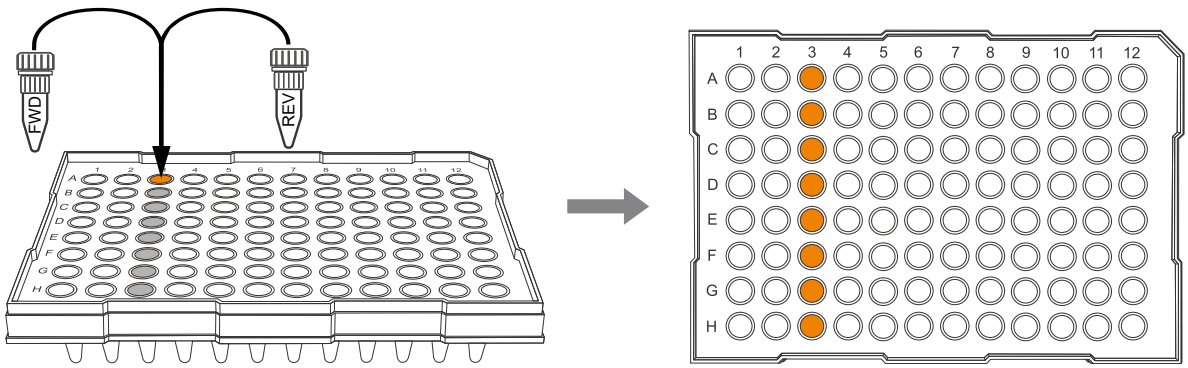
   As long as the precipitate goes back into solution, there is no effect on performance.

6. Add the following components to a single well of a 96-well PCR plate. Prepare a master mix without sample DNA for multiple reactions.
   - For optimal results, to achieve 0.1% LOD, use 50 ng of input DNA.
   - If the amount of input DNA is less than 50 ng, use the maximum amount available. The minimum input per sample is 20 ng.
   - For FFPE samples, add the reagents to each well containing pretreated DNA.

IMPORTANT! Do not substitute CRC for 5X HD Enhancer.

Component	Volume	
	cfTNA, cfDNA, or gDNA	Pretreated FFPE DNA[1]
4X Ion AmpliSeq™ HD Amplification Mix	7.5 µL	7.5 µL
5X HD Enhancer	6.0 µL	6.0 µL
10X working panel FWD subpool	3.0 µL	3.0 µL
10X working panel REV subpool	3.0 µL	3.0 µL
20–60 ng DNA (cfTNA, cfDNA, or gDNA) 50 ng of DNA is optimal.	≤10.5 µL	—
Nuclease-free water	to 30 µL	—
Total volume	**30 µL**	**30 µL**

[1] Reagents or master mix are added to each well containing pretreated DNA.



Wells that contain DNA, 4X Ion AmpliSeq™ HD Amplification Mix, and 5X HD Enhancer only

Wells that have working panel pool FWD and working panel pool REV added

7. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.
   - Alternatively, mix the reaction by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film.
   - To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

Proceed immediately to "Amplify the targets—DNA panel" on page 41.

Prepare molecular tagging reactions—2-pool primer panel

IMPORTANT!
- Keep all reagents on ice or in a prechilled 4°C cold block during reaction setup.
- Do not combine 10X DNA working panel FWD and REV primer subpools for storage. DNA working panel FWD and REV primer subpools must remain separate until combined in step 6.
- 4X Ion AmpliSeq™ HD Amplification Mix and 5X HD Enhancer are viscous. Pipet slowly and mix thoroughly.
- For legacy protocols without using 5X HD Enhancer, see Appendix C, "Prepare Ion AmpliSeq™ HD DNA libraries using CRC".
- Treat FFPE DNA samples with UDG before use. See "Remove deaminated bases from FFPE DNA" on page 33.

IMPORTANT! Before performing the molecular tagging reaction, prepare 10X DNA working panel FWD and REV primer subpools. See "Prepare 10X DNA FWD and REV working subpools—tube format" on page 28 or "Prepare 10X DNA FWD and REV working subpools—384-well plate format" on page 29.

1. Thaw the following components on ice for at least 10 minutes before use.
   - DNA samples
   - 4X Ion AmpliSeq™ HD Amplification Mix
   - 5X HD Enhancer
   - 10X DNA working panel FWD and REV primer subpools

2. Incubate the 4X Ion AmpliSeq™ HD Amplification Mix at room temperature for 10 minutes.

3. Chill a 96-well plate on ice.
   - For FFPE DNA samples, if the treated samples were frozen, thaw the sealed plate with the frozen pretreated samples, centrifuge briefly to collect the contents, carefully remove the plate seal, then place the plate on ice or in a prechilled 4°C cold block.
   - For non-FFPE DNA samples, place a 96-well PCR plate on ice.

4. Ensure that the DNA samples, 5X HD Enhancer, and Panel FWD and REV primer subpools are completely thawed, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.

5. Ensure that the 4X Ion AmpliSeq™ HD Amplification Mix is completely thawed and that there is no visible precipitate, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents. If you see precipitate, incubate the 4X Ion AmpliSeq™ HD Amplification Mix at room temperature for 10–20 minutes, vortex to completely dissolve the precipitate, then centrifuge briefly to collect the contents.

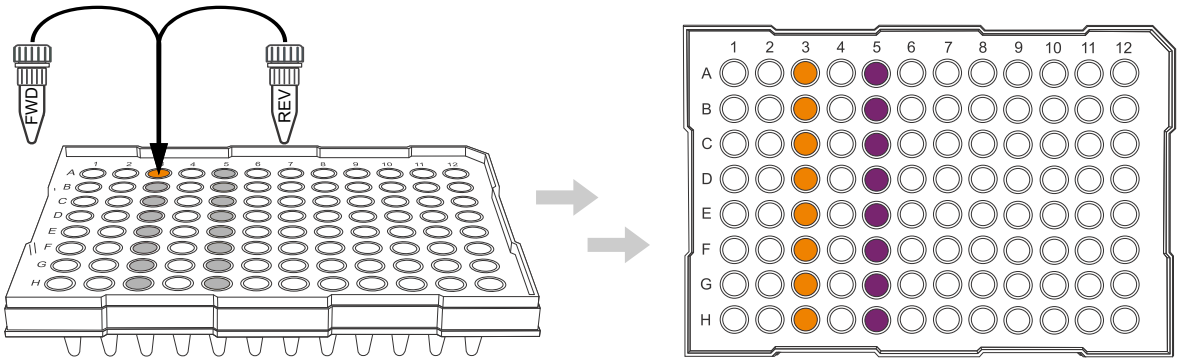
   As long as the precipitate goes back into solution, there is no effect on performance.

6. Add the following components to two wells of a 96-well PCR plate. For multiple reactions, prepare a master mix without the sample DNA.
   - For optimal results, to achieve 0.1% LOD, we recommend that you use 50 ng of input DNA.
   - If the amount of input DNA is less than 50 ng, we recommend that you use the maximum amount available. The minimum input per sample is 20 ng.
   - For 2-pool primer panels, prepare 2 wells, one for each pool. Each well requires 20–60 ng.
   - For FFPE samples, the reagents are added to each well containing pretreated DNA.

IMPORTANT! Do not substitute CRC for 5X HD Enhancer.

Component	Volume			
	Well 1, pool 1	Well 2, pool 2	Well 1, pool 1	Well 2, pool 2
	cfTNA, cfDNA, or gDNA		Pretreated FFPE DNA[1]	
4X Ion AmpliSeq™ HD Amplification Mix (purple cap)	3.75 µL	3.75 µL	3.75 µL	3.75 µL
5X HD Enhancer	3.0 µL	3.0 µL	3.0 µL	3.0 µL
10X working panel pool 1 FWD subpool	1.5 µL	—	1.5 µL	—
10X working panel pool 1 REV subpool	1.5 µL	—	1.5 µL	—
10X working panel pool 2 FWD subpool	—	1.5 µL	—	1.5 µL
10X working panel pool 2 REV subpool	—	1.5 µL	—	1.5 µL
20–60 ng DNA (cfTNA, cfDNA, gDNA) 50 ng of DNA is optimal.	≤5.25 µL	≤5.25 µL	—	—
Nuclease-free water	to 15 µL	to 15 µL	—	—
Total	**15 µL**	**15 µL**	**15 µL**	**15 µL**

[1] Reagents or master mix are added to each well containing pretreated DNA.



- Wells that contain DNA, 4X Ion AmpliSeq™ HD Amplification Mix, and 5X HD Enhancer only
- Wells that have working panel pool 1 FWD and working panel pool 1 REV added
- Wells that have working panel pool 2 FWD and working panel pool 2 REV added

7. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

Proceed immediately to “Amplify the targets—DNA panel” on page 41.

Prepare molecular tagging reactions—3-pool primer panel

IMPORTANT!

- Keep all reagents on ice or in a prechilled 4°C cold block during reaction setup.
- Do not combine 10X DNA working panel FWD and REV primer subpools for storage. DNA working panel FWD and REV primer subpools must remain separate until combined in step 6.
- 4X Ion AmpliSeq™ HD Amplification Mix and 5X HD Enhancer are viscous. Pipet slowly and mix thoroughly.
- For legacy protocols without using 5X HD Enhancer, see Appendix C, “Prepare Ion AmpliSeq™ HD DNA libraries using CRC”.
- Treat FFPE DNA samples with UDG before use. See “Remove deaminated bases from FFPE DNA” on page 33.

IMPORTANT! Before performing the molecular tagging reaction, prepare 10X DNA working panel FWD and REV primer subpools. See “Prepare 10X DNA FWD and REV working subpools—tube format” on page 28 or “Prepare 10X DNA FWD and REV working subpools—384-well plate format” on page 29.

1. Thaw the following components on ice for at least 10 minutes before use.
   - DNA samples
   - 4X Ion AmpliSeq™ HD Amplification Mix
   - 5X HD Enhancer
   - 10X DNA working panel FWD and REV primer subpools

2. Incubate the 4X Ion AmpliSeq™ HD Amplification Mix at room temperature for 10 minutes.

3. Chill a 96-well plate on ice.
   - For FFPE DNA samples, if the treated samples were frozen, thaw the sealed plate with the frozen pretreated samples, centrifuge briefly to collect the contents, carefully remove the plate seal, then place the plate on ice or in a prechilled 4°C cold block.
   - For non-FFPE DNA samples, place a 96-well PCR plate on ice.

4. Ensure that the DNA samples, 5X HD Enhancer, and Panel FWD and REV primer subpools are completely thawed, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.

5. Ensure that the 4X Ion AmpliSeq™ HD Amplification Mix is completely thawed and that there is no visible precipitate, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents. If you see precipitate, incubate the 4X Ion AmpliSeq™ HD Amplification Mix at room temperature for 10–20 minutes, vortex to completely dissolve the precipitate, then centrifuge briefly to collect the contents.

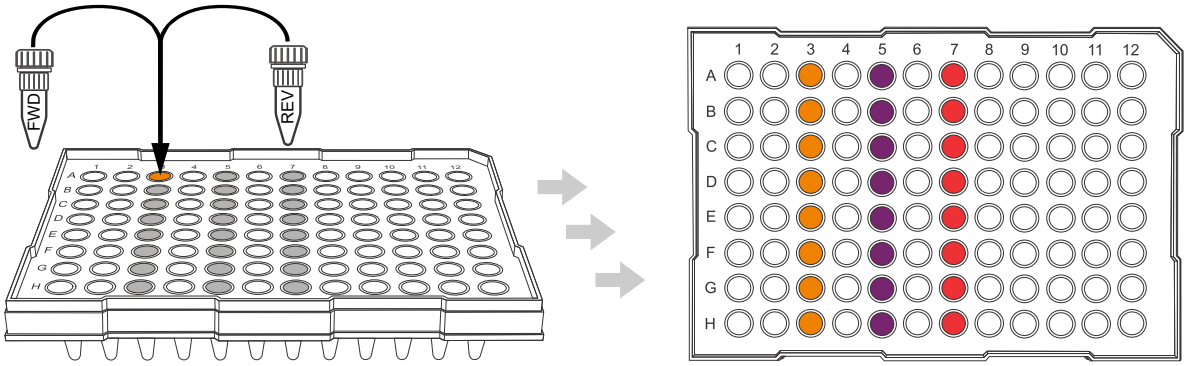
   As long as the precipitate goes back into solution, there is no effect on performance.

6. Add the following components to three wells of a 96-well PCR plate. For multiple reactions, prepare a master mix without the sample DNA.
   - For optimal results, to achieve 0.1% LOD, use 50 ng of input DNA.
   - If the amount of input DNA is less than 50 ng, use the maximum amount available. The minimum input per sample is 20 ng.
   - For FFPE samples, add the reagents to each well containing pretreated DNA.

   IMPORTANT! Do not substitute CRC for 5X HD Enhancer.

Component	Well 1, pool 1 (volume)	Well 2, pool 2 (volume)	Well 3, pool 3 (volume)
	cfTNA, cfDNA, or gDNA		
4X Ion AmpliSeq™ HD Amplification Mix	2.5 µL	2.5 µL	2.5 µL
5X HD Enhancer	2.0 µL	2.0 µL	2.0 µL
10X working panel pool 1 FWD subpool	1 µL	—	—
10X working panel pool 1 REV subpool	1 µL	—	—
10X working panel pool 2 FWD subpool	—	1 µL	—
10X working panel pool 2 REV subpool	—	1 µL	—
10X working panel pool 3 FWD subpool	—	—	1 µL
10X working panel pool 3 REV subpool	—	—	1 µL
20–60 ng DNA	≤3.5 µL	≤3.5 µL	≤3.5 µL
Nuclease-free water	to 10 µL	to 10 µL	to 10 µL
Total	**10 µL**	**10 µL**	**10 µL**
	FFPE DNA[1]		
4X Ion AmpliSeq™ HD Amplification Mix	2.5 µL	2.5 µL	2.5 µL
5X HD Enhancer	2.0 µL	2.0 µL	2.0 µL
10X working panel pool 1 FWD subpool	1 µL	—	—
10X working panel pool 1 REV subpool	1 µL	—	—
10X working panel pool 2 FWD subpool	—	1 µL	—
10X working panel pool 2 REV subpool	—	1 µL	—
10X working panel pool 3 FWD subpool	—	—	1 µL
10X working panel pool 3 REV subpool	—	—	1 µL
Total	**10 µL**	**10 µL**	**10 µL**

[1] Reagents or master mix are added to each well containing pretreated DNA.



- Wells that contain DNA, 4X Ion AmpliSeq™ HD Amplification Mix, and 5X HD Enhancer only
- Wells that have pool 1 FWD and pool 1 REV added
- Wells that have pool 2 FWD and pool 2 REV added
- Wells that have pool 3 FWD and pool 3 REV added

7. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

Proceed immediately to "Amplify the targets—DNA panel" on page 41.

Amplify the targets—DNA panel

1. Set the idle temperature of a thermocycler to 105°C for the lid and 25°C for the sample block.

   IMPORTANT! Do not preheat the thermocycler sample block.

2. Before loading the PCR plate, power on the thermocycler and wait for 10 minutes or until the block and lid reach the idle temperatures.

3. Place a MicroAmp™ Optical Film Compression Pad on the plate with molecular tagging reactions, then load the plate into the thermal cycler.

4. Run the following cycling program.

IMPORTANT! Run the program for only 3 cycles to ensure that exactly one unique molecular tag (UMT) is attached to each single strand of DNA for each target amplicon.

Stage	Step	Temperature	Time
3 cycles	Denature	99°C	30 seconds
	Anneal	64°C	2 minutes
		60°C	12 minutes
	Extend	66°C	2 minutes
		72°C	2 minutes
Hold	Final extension	72°C	2 minutes
Hold	—	4°C	≤2 hour

STOPPING POINT Proceed to "Partially digest tagged amplicons" on page 42 within 2 hours. If needed, you can store libraries at -20°C for 24 hours.

Partially digest tagged amplicons

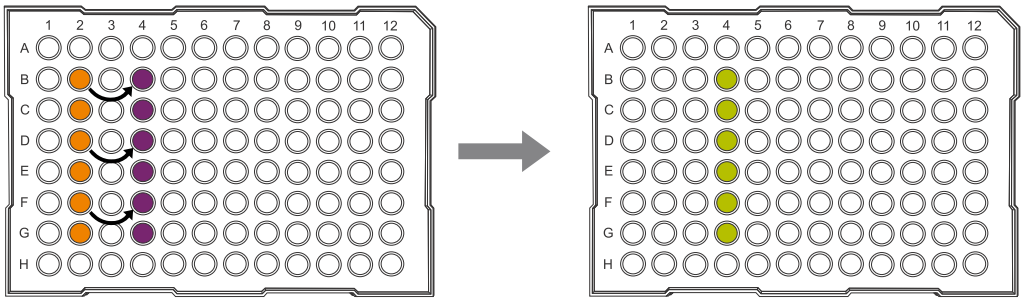
IMPORTANT! Do not substitute any assay components with reagents from other kits.

1. Set the idle temperature of a thermocycler to 105°C for the lid and 25°C for the sample block.

IMPORTANT! Do not preheat the thermocycler sample block.

2. Before loading the PCR plate, power on the thermocycler and wait for 10 minutes or until the block and lid reach the idle temperatures.
3. Thaw Ion AmpliSeq™ HD SUPA Reagent on ice.
4. If the sealed tagged amplicon plate was frozen, thaw on ice.
5. Centrifuge the sealed tagged amplicon plate briefly to collect contents, then place on ice or in a prechilled 4°C cold block.
6. Carefully remove the seal from the tagged amplicon plate.

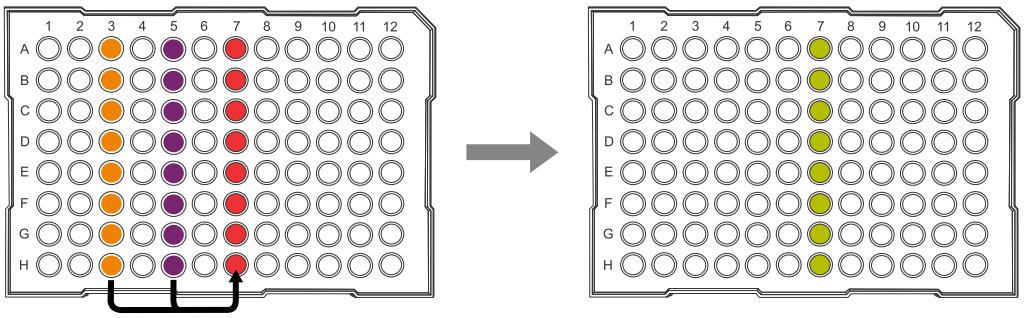
7. If you are using a 2-pool panel, for each sample, combine pool 1 and pool 2 by adding the pool 1 15-µL tagged amplification reaction to the pool 2 tagged amplification reaction well, then place the plate on ice or cold block.



- Wells with pool 1 FWD and pool 1 REV tagged amplicon reactions
- Wells with pool 2 FWD and pool 2 REV tagged amplicon reactions
- Wells with combined Primer pool 1 and Primer pool 2 tagged amplicon reactions

The total volume for each sample is 30 µL.

8. If you are using a 3-pool panel, for each sample, combine pool 1, pool 2, and pool 3 by adding the pool 1 and pool 2 10-µL tagged amplification reactions to the pool 3 tagged amplification reaction well, then place the plate on ice or cold block.



- Wells with pool 1 FWD and pool 1 REV target amplification reactions
- Wells with pool 2 FWD and pool 2 REV target amplification reactions
- Wells with pool 3 FWD and pool 3 REV target amplification reactions
- Wells with combined Primer pool 1, Primer pool 2, and Primer pool 3 target amplification reactions

The total volume for each sample is 30 µL.

9. Ensure that the Ion AmpliSeq™ HD SUPA Reagent has completely thawed with no visible ice present, vortex to mix, then centrifuge briefly to collect contents.

10. Add 5 µL of Ion AmpliSeq™ HD SUPA Reagent to each 1-pool panel reaction well or to each combined reaction well for 2-pool or 3-pool panel.

IMPORTANT!

- FuPa Reagent is *not* a substitute for the Ion AmpliSeq™ HD SUPA Reagent.
- Ion AmpliSeq™ HD SUPA Reagent is viscous. Pipet slowly.

11. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.

    To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

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

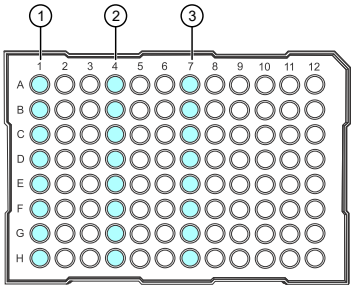
Temperature	Time
30°C	15 minutes
50°C	15 minutes
55°C	15 minutes
25°C	10 minutes
98°C	2 minutes
4°C	Hold (≤2 hour)

IMPORTANT! Not a stopping point. Immediately proceed to "Amplify the library with barcoded primers" on page 44.

Amplify the library with barcoded primers

Barcode primers from the Ion AmpliSeq™ HD Dual Barcode Kit 1–24 are required for library preparation when sequencing one or more libraries per sequencing chip. If you are sequencing multiple libraries on a single chip, you must use different barcode primers with each library.

A blue dye is added to the barcode primers to help identify wells that contain primers. Columns 1, 4, and 7 in a 96-well plate contain primers in liquid format. Briefly centrifuge the plate before use.



① Column 1 contains barcode primers 1–8 in ascending order from top to bottom.
② Column 4 contains barcode primers 9–16 in ascending order from top to bottom.
③ Column 7 contains barcode primers 17–24 in ascending order from top to bottom.

IMPORTANT! When preparing libraries, we recommend that you alternate barcodes between consecutive sequencing runs to prevent carryover contamination. For more information, see "Procedural guidelines" on page 19.

1. Set the idle temperature of a thermocycler to 105°C for the lid and 25°C for the sample block.

   IMPORTANT! Do not preheat thermocycler sample block.

2. Before loading the PCR plate, power on the thermocycler and wait for 10 minutes or until the block and lid reach the idle temperatures.

3. Thaw the Ion AmpliSeq™ HD Dual Barcode Kit 1–24 plate (barcode plate) on ice.

4. Ensure that the Ion AmpliSeq™ HD Dual Barcode Kit 1–24 plate is completely thawed with no visible ice present, then briefly centrifuge the barcode plate to collect contents.

5. Remove the partially digested tagged amplicon reaction plate from the thermal cycler, centrifuge briefly to collect the contents, then place on ice or cold block.

6. Carefully remove the adhesive film from the partially digested tagged amplicon reaction plate.

7. Add 4 µL of the selected barcode primers from the Ion AmpliSeq™ HD Dual Barcode Kit 1–24 to each partially digested tagged amplicon reaction well. Use a 1 mL pipette tip to punch a hole on the foil above the barcode well to enable the pipette to access the barcodes.

   IMPORTANT! Do not peel the foil cover on the barcode plate, as removing the foil cover can lead to barcode cross-contamination.

8. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.

9. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate into the thermocycler, then run the following program.

Stage	Step	Temperature	Time
5 cycles	Denature	99°C	20 seconds
	Anneal	60°C	40 seconds
	Extend	72°C	40 seconds
12–15 cycles[1]. See Table 4	Denature	99°C	20 seconds
	Anneal/extend	66°C	60 seconds
Hold	Final extension	72°C	2 minutes
Hold	—	4°C	Indefinite

[1] For FFPE DNA, using 17 cycles can improve library yields.

Table 4 Recommended number of amplification cycles

Total number of amplicons in the panel (1-, 2-, or 3-pool panels)	Number of cycles
12–399	15
400–1,000	14
1,001–2,000	13
2,001–5,000	12

10. Briefly centrifuge the plate to collect contents.

STOPPING POINT Library amplification products can be stored at 4°C overnight on the thermal cycler. Store at –20°C for up to 3 days.

Purify the DNA 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.

Before you begin:

- Prepare 70% ethanol in nuclease-free water (650 µL × number of samples) fresh daily.
- If the library reaction plate was frozen, thaw the plate at room temperature.
- Vortex the sealed library reaction plate for 10 seconds to mix, then centrifuge briefly to collect the contents.

Purify the library with MagMAX™ Pure Bind Beads

1. Carefully remove the plate seal from the library reaction plate, then add 10 µL of Low TE to each library well.
2. Vortex the MagMAX™ Pure Bind Beads for 30 seconds to resuspend the beads.
3. Add 39 µL (1X sample volume before Low TE addition) of the MagMAX™ Pure Bind Beads to each library well, then pipet up and down 10 times to thoroughly mix the bead suspension with the library mix. Avoid generating excessive bubbles.

   Visually inspect each well to ensure that the mixture is homogeneous.
4. Incubate the mixture for 5 minutes at room temperature.

5. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution clears.
6. Carefully remove, then discard the supernatant without disturbing the pellet.
7. Seal the plate with MicroAmp™ Clear Adhesive Film, then briefly centrifuge the plate to collect the contents.
8. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, carefully remove the plate seal, then use a 20-µL pipette to remove remaining liquid in each library well.
9. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet 10 times to wash the beads.
10. Carefully remove, then discard the supernatant without disturbing the pellet.
11. Repeat step 9 and step 10 one more time.
12. Seal the plate with MicroAmp™ Clear Adhesive Film, then briefly centrifuge the plate to collect the contents.
13. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, carefully remove the plate seal, then use a 20-µL pipette to remove remaining liquid in each library well.
14. 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.
15. Remove the plate from the magnet, add 52 µL of Low TE buffer to each library well, then resuspend the MagMAX™ Pure Bind Beads by pipetting up and down 10 times to fully disperse the beads into the solution.

    Visually inspect each well to ensure that the mixture is homogeneous.
16. Incubate the plate at room temperature for 5 minutes.
17. Place the plate back in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution clears.
18. Transfer 50 µL of the purified library into a new well.

Further purification with MagMAX™ Pure Bind Beads

1. Remove the plate from the magnet, add 50 µL (1X sample volume) of MagMAX™ Pure Bind Beads to each library well, then pipet up and down 10 times to thoroughly mix the bead suspension with the library. Avoid generating excessive bubbles.

    Visually inspect each well to ensure that the mixture is homogeneous.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution is clear.

4. Carefully remove, then discard the supernatant without disturbing the pellet.
5. Seal the plate with MicroAmp™ Clear Adhesive Film, then briefly centrifuge the plate to collect the contents.
6. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, carefully remove the plate seal, then use a 20-µL pipette to remove remaining liquid in each library well.
7. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet 10 times to wash the beads.
8. Carefully remove, then discard the supernatant without disturbing the pellet.
9. Repeat step 7 and step 8 one more time.
10. Seal the plate with MicroAmp™ Clear Adhesive Film, then briefly centrifuge the plate to collect the contents.
11. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, carefully remove the plate seal, then use a 20-µL pipette to remove remaining liquid in each library well.
12. 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.
13. Remove the plate from the magnet, add 52 µL of Low TE buffer to each well, then pipet up and down 10 times to resuspend the MagMAX™ Pure Bind Beads.
14. Incubate the plate at room temperature for 5 minutes.
15. Place the plate back in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution is clear.
16. Transfer 50 µL of the cleared library solution into a new tube or well in a plate.

This is your library product.

Library quantification

Quantify the DNA library using one of the following options.

- Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument (see Chapter 8, “Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument”).

  IMPORTANT! We recommend that you quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument when you use a different sample type or a panel for the first time.

- Quantify the library by qPCR (see “Quantify the library by qPCR” on page 69).
- Use the Ion Library Equalizer™ Kit to normalize library concentration (see “Equalize the library” on page 72).

7 Prepare Ion AmpliSeq™ HD RNA libraries

IMPORTANT! This protocol is designed for use with 1-pool RNA primers panels. It is not compatible for use wih panels that contain both DNA and RNA primers.

For legacy protocols without using 5X HD Enhancer, see Appendix D, “Prepare Ion AmpliSeq™ HD RNA libraries without using 5X HD Enhancer”.

Guidelines for RNA isolation, quantification, and input

- For recommended isolation kits, see “Recommended materials and equipment” on page 17.
- FFPE RNA or total RNA are compatible.
- We recommend that you use the Qubit™ RNA HS Assay Kit (Cat. No. Q32852) for quantifying RNA.
- Each reverse transcription reaction requires 20 ng of RNA (≥2.9 ng/µL).
- In general, library yield from high quality RNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.
- If the library yield is below 100 pM, you can still proceed to sequencing by using a proportionally larger volume of the library when combining libraries or during template preparation.

Before each use of the kit

- Thaw the 4X Ion AmpliSeq™ HD Amplification Mix, 5X HD Enhancer, and Ion AmpliSeq™ HD SUPA Reagent, and Ion AmpliSeq™ HD Dual Barcode Kit 1–24 on ice. Keep on ice during the procedure.
- Do not dilute RNA 10X stock panel FWD and REV subpools. The RNA stock FWD and REV subpools are the 10X RNA working panels.

Reverse transcribe the RNA

IMPORTANT! Keep all reagents on ice or in a prechilled 4°C cold block during reaction setup.

Materials required—Ion Torrent™ NGS Reverse Transcription Kit (Cat. No. A45003)

- 5X Reaction Buffer
- 10X RT Enzyme Mix

1. Set the idle temperature of a thermocycler to 105°C for the lid and 25°C for the sample block.

   IMPORTANT! Do not preheat the thermocycler sample block.

2. Before loading the PCR plate, power on the thermocycler and wait for 10 minutes or until the block and lid reach the idle temperatures.

3. Thaw the following components on ice.
   - 5X Reaction Buffer
   - 10X RT Enzyme Mix
   - RNA samples

4. If the RNA was prepared from FFPE tissue and not previously heat-treated, heat at 80°C for 10 minutes, then cool on ice for at least for 5 minutes before use.

5. Place a 96-well plate (reverse transcription plate) on ice or in a prechilled 4°C cold block.

6. Ensure that 5X Reaction Buffer, 10X RT Enzyme Mix, and RNA samples are completely thawed with no visible ice present, vortex to mix, then centrifuge briefly to collect contents.

7. For each sample, add the following components to a single well of the reverse transcription 96-well PCR plate. For multiple reactions, prepare a master mix without sample RNA.

Component	Volume
5X Reaction Buffer	2.0 µL
10X RT Enzyme Mix	1.0 µL
20 ng RNA	≤7.0 µL
Low TE	to 10.0 µL
Total	**10.0 µL**

8. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.
   - Alternatively, mix the reaction by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film.
   - To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.
9. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate in the thermal cycler, then run the following program.

Temperature	Time
25°C	10 minutes
50°C	10 minutes
85°C	5 minutes
4°C	Hold (≤1 hour)

10. Briefly centrifuge the plate to collect the contents.

IMPORTANT! Not a stopping point. Immediately proceed to "Prepare cDNA molecular tagging reactions—RNA panel" on page 51.

Prepare cDNA molecular tagging reactions—RNA panel

IMPORTANT!
- Keep all reagents on ice or in a prechilled 4°C cold block during reaction setup.
- Do not combine RNA FWD and REV primer subpools for storage. Primer subpools must remain separate until combined for the target amplification reaction.
- 4X Ion AmpliSeq™ HD Amplification Mix and 5X HD Enhancer are viscous. Pipet slowly and mix thoroughly.
- Do not substitute CRC for 5X HD Enhancer.
- To use CRC, see Appendix C, "Prepare Ion AmpliSeq™ HD DNA libraries using CRC" or Appendix D, "Prepare Ion AmpliSeq™ HD RNA libraries without using 5X HD Enhancer".
- Do not dilute RNA 10X stock panel FWD and REV subpools. The RNA stock FWD and REV subpools are the 10X RNA working panels.

1. Thaw the following components on ice for at least 10 minutes before use.
   - 4X Ion AmpliSeq™ HD Amplification Mix
   - 5X HD Enhancer
   - 10X RNA stock panel FWD and REV primer subpools
2. Incubate the 4X Ion AmpliSeq™ HD Amplification Mix at room temperature for 10 minutes.
3. Place the reverse transcription plate on ice or in a prechilled 4°C cold block, then carefully remove the plate seal.

4. Ensure that the 5X HD Enhancer and Panel FWD and REV primer subpools are completely thawed, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.

5. Ensure that there is no precipitation in the 4X Ion AmpliSeq™ HD Amplification Mix. If you see precipitate, incubate the mix at room temperature for 10–20 minutes, then vortex to completely dissolve the precipitate.

   As long as the precipitate goes back into solution, there is no effect on performance.

6. Add the following components to the well of each reverse transcription reaction. Prepare a master mix without sample cDNA for multiple reactions.

Component	Volume
Reverse transcription reaction	10 µL[1]
4X Ion AmpliSeq™ HD Amplification Mix	7.5 µL
5X HD Enhancer	6.0 µL
10X RNA FWD subpool	3.0 µL
10X RNA REV subpool	3.0 µL
Nuclease-free water	0.5 µL
Total	**30 µL**

[1] cDNA volume in each target amplification reaction is the entire volume of the reverse transcription reaction.

7. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.
   - Alternatively, mix the reaction by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film.
   - To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

Proceed immediately to "Amplify the targets—RNA panel" on page 52

Amplify the targets—RNA panel

1. Set the idle temperature of a thermocycler to 105°C for the lid and 25°C for the sample block.

   IMPORTANT! Do not preheat the thermocycler sample block.

2. Before loading the PCR plate, power on the thermocycler and wait for 10 minutes or until the block and lid reach the idle temperatures.

3. Place a MicroAmp™ Optical Film Compression Pad on the plate with molecular tagging reactions, then load the plate into the thermal cycler.

4. Run the following cycling program.

IMPORTANT! Run the program for only 3 cycles to ensure that exactly one unique molecular tag (UMT) is attached to each single strand of DNA for each target amplicon.

Stage	Step	Temperature	Time
3 cycles	Denature	99°C	30 seconds
	Anneal	64°C	2 minutes
		60°C	12 minutes
	Extend	66°C	2 minutes
		72°C	2 minutes
Hold	Final extension	72°C	2 minutes
Hold	—	4°C	≤2 hour

STOPPING POINT Proceed to “Partially digest tagged amplicons” on page 42 within 2 hours. If needed, you can store libraries at -20°C for 24 hours.

Partially digest tagged amplicons

IMPORTANT! Do not substitute any assay components with reagents from other kits.

1. Set the idle temperature of a thermocycler to 105°C for the lid and 25°C for the sample block.

IMPORTANT! Do not preheat the thermocycler sample block.

2. Before loading the PCR plate, power on the thermocycler and wait for 10 minutes or until the block and lid reach the idle temperatures.
3. Thaw Ion AmpliSeq™ HD SUPA Reagent on ice.
4. If the sealed tagged amplicon plate was frozen, thaw on ice.
5. Centrifuge the sealed tagged amplicon plate briefly to collect the contents, then place on ice or in a prechilled 4°C cold block.
6. Carefully remove the seal from the tagged amplicon plate.
7. Add 5 µL of Ion AmpliSeq™ HD SUPA Reagent to each library reaction well.

IMPORTANT!

- FuPa Reagent is NOT a substitute for the Ion AmpliSeq™ HD SUPA Reagent.
- Ion AmpliSeq™ HD SUPA Reagent is viscous. Pipet slowly.

8. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

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

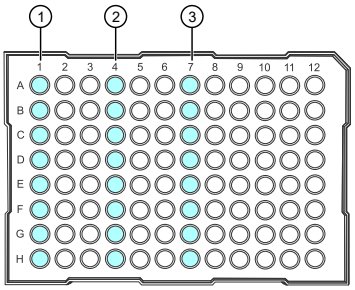
Temperature	Time
30°C	15 minutes
50°C	15 minutes
55°C	15 minutes
25°C	10 minutes
98°C	2 minutes
4°C	Hold (≤1 hour)

IMPORTANT! Not a stopping point. Immediately proceed to "Amplify the library with barcoded primers" on page 54.

Amplify the library with barcoded primers

Ion AmpliSeq™ HD Dual Barcode Kit 1–24 primers are provided ready-to-use with different barcode primers per well. These primers are required for library preparation when sequencing one or multiple libraries per sequencing chip. If you are sequencing multiple libraries on a single chip, you must use different barcode primers with each library.

A blue dye is added to the barcode primers to help identify wells that contain primers. Columns 1, 4, and 7 in a 96-well plate contain primers in liquid format. Briefly centrifuge the plate before use.



① Column 1 contains barcode primers 1–8 in ascending order from top to bottom.
② Column 4 contains barcode primers 9–16 in ascending order from top to bottom.
③ Column 7 contains barcode primers 17–24 in ascending order from top to bottom.

IMPORTANT! When preparing libraries, we recommend that you alternate barcodes between consecutive sequencing runs to prevent carryover contamination. For more information, see "Procedural guidelines" on page 19.

1. Set the idle temperature of a thermocycler to 105°C for the lid and 25°C for the sample block.

   IMPORTANT! Do not preheat thermocycler sample block.

2. Before loading the PCR plate, power on the thermocycler and wait for 10 minutes or until the block and lid reach the idle temperatures.

3. Thaw the Ion AmpliSeq™ HD Dual Barcode Kit 1–24 plate (barcode plate) on ice.

4. Ensure that the Ion AmpliSeq™ HD Dual Barcode Kit 1–24 plate is completely thawed with no visible ice present, then briefly centrifuge the barcode plate to collect contents.

5. Remove the partially digested tagged amplicon reaction plate from the thermal cycler, centrifuge briefly to collect the contents, then place on ice or cold block.

6. Carefully remove the adhesive film from the partially digested tagged amplicon reaction plate.

7. Add 4 µL of the selected barcode primers from the Ion AmpliSeq™ HD Dual Barcode Kit 1–24 to each partially digested tagged amplicon reaction well. Use a 1 mL pipette tip to punch a hole on the foil above the barcode well to enable the pipette to access the barcodes.

   IMPORTANT! Do not peel the foil cover on the barcode plate, as removing the foil cover can lead to barcode cross-contamination.

8. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex for 10 seconds to mix, then centrifuge briefly to collect the contents.

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

Stage	Step	Temperature	Time
5 cycles	Denature	99°C	20 seconds
	Anneal	60°C	40 seconds
	Extend	72°C	40 seconds
17 cycles	Denature	99°C	20 seconds
	Anneal/Extend	66°C	60 seconds
Hold	Final extension	72°C	2 minutes
Hold	—	4°C	Indefinite

10. Briefly centrifuge the plate to collect the contents in the bottom of the wells.

STOPPING POINT Library amplification products can be stored at 4°C overnight on the thermal cycler. For longer periods, store at –20°C.

Purify the RNA 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.

Before you begin:

- Prepare 70% ethanol in nuclease-free water (650 µL × number of samples) fresh daily.
- If the library reaction plate was frozen, thaw the plate at room temperature.
- Vortex the sealed library reaction plate for 10 seconds to mix, then centrifuge briefly to collect the contents.

Purify the library with MagMAX™ Pure Bind Beads

1. Carefully remove the plate seal from the library reaction plate, then add 10 µL of Low TE to each library well.
2. Vortex the MagMAX™ Pure Bind Beads for 30 seconds to resuspend the beads.
3. Add 39 µL (1X sample volume before Low TE addition) of the MagMAX™ Pure Bind Beads to each library well, then pipet up and down 10 times to thoroughly mix the bead suspension with the library mix. Avoid generating excessive bubbles.

   Visually inspect each well to ensure that the mixture is homogeneous.
4. Incubate the mixture for 5 minutes at room temperature.
5. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution clears.
6. Carefully remove, then discard the supernatant without disturbing the pellet.
7. Seal the plate with MicroAmp™ Clear Adhesive Film, then briefly centrifuge the plate to collect the contents.
8. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, carefully remove the plate seal, then use a 20-µL pipette to remove remaining liquid in each library well.
9. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet 10 times to wash the beads.
10. Carefully remove, then discard the supernatant without disturbing the pellet.

11. Repeat step 9 and step 10 one more time.
12. Seal the plate with MicroAmp™ Clear Adhesive Film, then briefly centrifuge the plate to collect the contents.
13. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, carefully remove the plate seal, then use a 20-µL pipette to remove remaining liquid in each library well.
14. 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.
15. Remove the plate from the magnet, add 52 µL of Low TE buffer to each library well, then resuspend the MagMAX™ Pure Bind Beads by pipetting up and down 10 times to fully disperse the beads into the solution.

    Visually inspect each well to ensure that the mixture is homogeneous.
16. Incubate the plate at room temperature for 5 minutes.
17. Place the plate back in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution clears.
18. Transfer 50 µL of the purified library into a new well.

Further purification with MagMAX™ Pure Bind Beads

1. Remove the plate from the magnet, add 50 µL (1X sample volume) of MagMAX™ Pure Bind Beads to each library well, then pipet up and down 10 times to thoroughly mix the bead suspension with the library. Avoid generating excessive bubbles.

    Visually inspect each well to ensure that the mixture is homogeneous.
2. Incubate the mixture for 5 minutes at room temperature.
3. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution is clear.
4. Carefully remove, then discard the supernatant without disturbing the pellet.
5. Seal the plate with MicroAmp™ Clear Adhesive Film, then briefly centrifuge the plate to collect the contents.
6. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, carefully remove the plate seal, then use a 20-µL pipette to remove remaining liquid in each library well.
7. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet 10 times to wash the beads.
8. Carefully remove, then discard the supernatant without disturbing the pellet.
9. Repeat step 7 and step 8 one more time.

10. Seal the plate with MicroAmp™ Clear Adhesive Film, then briefly centrifuge the plate to collect the contents.
11. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, carefully remove the plate seal, then use a 20-µL pipette to remove remaining liquid in each library well.
12. 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.
13. Remove the plate from the magnet, add 52 µL of Low TE buffer to each well, then pipet up and down 10 times to resuspend the MagMAX™ Pure Bind Beads.
14. Incubate the plate at room temperature for 5 minutes.
15. Place the plate back in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution is clear.
16. Transfer 50 µL of the cleared library solution into a new tube or well in a plate.

This is your library product.

Library quantification

Quantify the RNA library using one of the following options.

- Option 1—Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument (see Chapter 8, “Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument”).

  IMPORTANT! We recommend that you quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument when you use a different sample type or a panel for the first time.

- Option 2—Quantify the library by qPCR (see “Quantify the library by qPCR” on page 69).
- Option 3—Use the Ion Library Equalizer™ Kit to normalize library concentration (see “Equalize the library” on page 72).

8 Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument

IMPORTANT!

- When using new Ion AmpliSeq™ HD panels or using new sample types, we recommend that you quantify Ion AmpliSeq™ HD libraries with the Agilent™ 2100 Bioanalyzer™ Instrument.
- The qPCR library quantification method is recommended for Ion AmpliSeq™ HD libraries that have been determined to have low levels of primer dimers.
- When library yields are consistently above 0.5 nM, the Ion Library Equalizer™ Kit can be used reliably. If sample quality or quantity is variable or unknown (such as RNA from FFPE tissue, or Direct FFPE DNA), the Agilent™ 2100 Bioanalyzer™ Instrument quantification method may provide greater accuracy of library yield and the resulting number of sequencing reads.

We recommend that you determine library concentration using the Agilent™ 2100 Bioanalyzer™ Instrument, because this method also allows you to evaluate the library primer-dimer profile. Libraries typically have yields of 1,000–40,000 pM. Yield is not indicative of library quality, and libraries below 1,000 pM can still provide good quality sequences. RNA libraries can have yields less than 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.

Alternatively, the Ion Library TaqMan™ Quantitation Kit can be used to quantify amplified libraries. For more information, see “Quantify the library by qPCR” on page 69.

Quantify the library and calculate the dilution factor

Analyze 1 µL of the library on the Agilent™ 2100 Bioanalyzer™ Instrument with the Agilent™ High Sensitivity DNA Kit (Cat. No. 5067-4626). If the library concentration is over 20,000 pM, dilute the library 1:10 and repeat the quantification to obtain a more accurate measurement.

1. Determine the molar concentration of the library using the Bioanalyzer™ software. Ensure that the upper and lower marker peaks are identified and assigned correctly. Follow the instructions of the manufacturer to perform a region analysis (smear analysis). Briefly:
    a. In the **Contexts** panel, select the **Data** icon, then view the electropherogram of the sample to be quantified.

b. Select the **Region Table** tab below, then create a region that spans the desired amplicon library peaks. Correct the baseline, if needed.
   - Generally, the expected amplicon library size falls within the 220-bp to 320-bp range.
   - To calculate the expected amplicon library size range for the desired panel, add 150 bp to the design size range to account for the adapter sequence length. Peaks in the 50 bp to 220 bp size range could be primer dimer byproducts. Peaks in the 320 bp to 700 bp range could be superamplicon byproducts.

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

   Generally, the calculation uses amplicon library size within the 220-bp to 320-bp range.

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

   IMPORTANT! To ensure that each library has similar on target sequencing reads when combined with other libraries, use only amplicon library concentration values in the 220–320 bp range to calculate the library dilution factor.

   For example:
   - The library concentration is 3,000 pM.
   - The dilution factor is 3,000 pM/100 pM = 30.
   - Therefore, 10 µL of library mixed with 290 µL of Low TE (1:30 dilution) yields approximately 100 pM.

3. Dilute the library to 100 pM, then proceed to Chapter 9, “Combine libraries” or store libraries as described in “Store libraries” on page 60.

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.

9 Combine libraries

Compatible kits, chips, and instruments

Proceed to template preparation and sequencing using the following kits. Use 25 µL of the 100-pM library or combined library per chip for templating on the Ion Chef™ System.

Chip	Template system	Sequencer	Kit	User guide
Ion 530™ Chip	Ion Chef™	Ion GeneStudio™ S5 Series	Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef (Cat. Nos. A34461, A34019)	*Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide* (Pub. No. MAN0016854)
Ion 540™ Chip			Ion 540™ Kit – Chef (Cat. No. A30011)	*Ion 540™ Kit – Chef User Guide* (Pub. No. MAN0010851)
Ion 550™ Chip[1]			Ion 550™ Kit – Chef (Cat. No. A34541)	*Ion 550™ Kit – Chef User Guide* (Pub. No. MAN0017275)

[1] The Ion 550™ Chip is compatible only with the Ion GeneStudio™ S5 Plus and Ion GeneStudio™ S5 Prime model sequencers.

Note: These kits are NOT compatible with Ion PGM™ System and Ion Proton™ System.

Guidelines for chip selection

Use the following guidelines to select the appropriate chip for sequencing your Ion AmpliSeq™ HD library. The DNA type and amplicon size range is determined at the time of custom Ion AmpliSeq™ HD panel creation in Ion AmpliSeq™ Designer. For more information about designing and ordering custom Ion AmpliSeq™ HD panels, see *Ion AmpliSeq™ Designer Help* (available at **AmpliSeq.com**).

Chip	DNA sample type[1]	Amplicon size range
Ion 530™ Chip	cfDNA/FFPE ("short" designs) *and* FFPE ("long" designs)	75–125 bp *and* 125–175 bp
Ion 540™ Chip	cfDNA/FFPE ("short" designs) only	75–125 bp
Ion 550™ Chip		

[1] Selected at the time of design creation in Ion AmpliSeq™ Designer (**AmpliSeq.com**).

Combine libraries—DNA panels

Multiple barcoded libraries prepared using the same DNA panel can be sequenced on a single chip by combining equal volumes of each library before template preparation.

The number of DNA libraries that can be combined on one chip depends on the following factors.

- Quantity of input DNA
- Number of amplicons in a panel
- Chip type

1. Dilute all individual barcoded libraries to 100-pM concentration.

2. Calculate the number of libraries that can be combined.

   $S = T / (D \times A)$

   where

   - S = Number of libraries per chip
   - T = Reads per chip
   - D = Average read depth per amplicon
   - A = Number of amplicons per panel

Table 5 Chip data for the Ion 530™ Chip, Ion 540™ Chip, and Ion 550™ Chip

Ion sequencing chip	Ion 530™ Chip	Ion 540™ Chip	Ion 550™ Chip
Reads per chip (M)	15–20	60–80	100–130
Target LOD	0.1%	0.1%	0.1%
Input DNA	20–60 ng	20–60 ng	20–60 ng
Recommended average depth per amplicon	70,000X	70,000X	70,000X

For example—The number of samples for an Ion AmpliSeq™ HD panel with 200 amplicons when aiming at 0.1% LOD starting from 20–60 ng of input DNA for sequencing on an Ion 540™ Chip.

- T = 60,000,000 reads per Ion 540™ Chip
- A = 200 amplicons
- D = 70,000 reads
- S = 60,000,000 / (70,000 * 200) = 4.3 = 4 libraries per chip

3. Based on the calculation in step 2, combine 10 µL or more of each library in a single 1.5-mL Eppendorf™ LoBind™ tube.

   The combined library mix must be at least 30 µL.

4. After adding the last library, pipet up and down 5 times to mix, then centrifuge briefly to collect in the contents.

STOPPING POINT If templating the same day, store the combined library pool at 4°C before use. For longer term storage, store at –20°C.

Combine libraries—RNA panels

Multiple barcoded libraries prepared using the same RNA panel can be sequenced on a single chip by combining equal volumes of each library before template preparation.

The number of RNA libraries that can be combined on one chip depends on the following factors:

- Quantity of input RNA
- Chip type

1. Dilute all individual barcoded libraries to 100-pM concentration.

2. Calculate the number of libraries that can be combined.

   S = T / R

   where

   - S = Number of libraries per chip
   - T = Reads per chip
   - R = Reads per RNA library—Depends on quantity of RNA input and the RNA penal type. For 20 ng of RNA input, 2.5 million reads are needed per RNA fusion library.

   For more information about calculating average read depth, contact support.

   For example—The number of samples for an Ion AmpliSeq™ HD RNA fusion panel using 20 ng of input RNA for sequencing on an Ion 540™ Chip.

   - T = 60,000,000 reads per Ion 540™ Chip
   - DR = 2.5 million reads
   - S = 60,000,000 / (2,500,000) = 24 libraries per chip

3. Based on the calculation in step 2, combine 5 µL or more of each library in a single 1.5-mL Eppendorf™ LoBind™ tube.

   The combined library mix must be at least 30 µL.

4. After adding the last library, pipet up and down 5 times to mix, then centrifuge briefly to collect in the contents.

STOPPING POINT If templating the same day, store the combined library pool at 4°C before use. For longer term storage, store at –20°C.

Troubleshooting and FAQs

Visit our online Support Centers and FAQ database for tips and tricks for conducting your experiment, troubleshooting information, and FAQs. The online FAQ database is frequently updated to ensure accurate and thorough content.

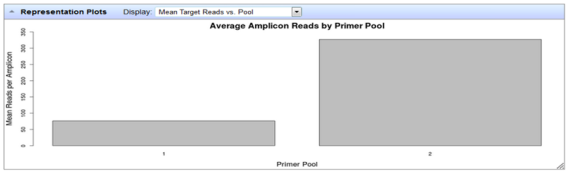
- For the Next–Generation Sequencing Support Center: **thermofisher.com/ngssupport**
- For FAQs for this product: **http://thermofisher.com/A57283faqs**
- To browse the FAQ database and search using keywords: **thermofisher.com/faqs**

Troubleshooting

Library yield and quantification

Observation	Possible cause	Recommended action
Library concentration is low—general **Details:** (Library concentration is NOT indicative of quality.)	Sample DNA or RNA was mis-quantified.	Requantify sample with Qubit™ 2.0 Fluorometer or later.
	Sample DNA or RNA quality was low.	Add more DNA/RNA or increase library amplification cycles.
	PCR or digestion was inefficient.	Ensure proper dispensing and mixing of viscous components at each step.
	MagMAX™ Pure Bind Beads were over-dried.	Do not dry the MagMAX™ Pure Bind Beads more than 5 minutes.
	FFPE RNA was not heat treated before reverse transcription.	Heat FFPE RNA at 80°C for 10 minutes, then cool to room temperature before reverse transcribing.
Library concentration is too high	Sample DNA or RNA was misquantified.	Requantify sample with Qubit™ 2.0 Fluorometer or later.
	More than 100 ng of sample DNA/RNA was used.	Add less DNA/RNA, or decrease library amplification cycles.
Primer-dimers are observed in Agilent™ 2100 Bioanalyzer™ results	Primer dimer formed during reaction setup.	Make sure to perform an additional round of MagMAX™ Pure Bind Beads purification of the library (see "Purify the DNA library" on page 46 and in "Purify the RNA library" on page 56).
		For challenging primer panels that produce excessive amounts of primer dimer, use the CRC reagent to your target amplification reaction to reduce the primer dimer product (see page 34 and 51).
		Amplify the targets using the alternative cycling option B as described in "Amplify the targets—DNA panel" on page 41.
		For significant primer-dimer content (~30%–70%), reduce the concentration of panel primer in target amplification reactions: • For panels with less than 500 primer pairs per pool, you can reduce the primer concentration by 5X, down to 10 nM. • For panels with 501–1,000 primer pairs per pool, you can reduce the primer concentration by 2.5X, down to 10 nM. • For panels with more than 1,000 primer pairs per pool, you can reduce the primer concentration down to 5 nM.

Low amplicon uniformity (DNA only)

Observation	Possible cause	Recommended action
Short amplicons are under-represented	Library purification was poor due to insufficient amount of MagMAX™ Pure Bind Beads.	Vortex the MagMAX™ Pure Bind Beads thoroughly before use and be sure to dispense the full volume.
Pool representation is not balanced  **Example of pool imbalance.** Within the coverageAnalysis plugin, mean read depth per primer pool is plotted for a 2-pool Ion AmpliSeq™ panel. In this example, Primer Pool 1 has approximately one quarter the reads of Primer Pool 2.	Amount of DNA in target amplification reactions varied.	Ensure that the same DNA input is added to the reaction for each primer pool.
	Pipetting is inaccurate when pools are combined after target amplification reaction.	Centrifuge the plate after the target amplification reaction. Ensure that the entire volume of the pool is removed and combined with the other pool.

Other

Observation	Possible cause	Recommended action
Barcode representation is uneven	Library quantification was inaccurate.	Repeat library quantification to confirm library yield.
	Library combination was inaccurate.	Dilute libraries to 100 pM, then combine equal volumes.
Percentage of polyclonal ISPs is high (>40%)	Library input was too high.	Decrease amount of library added to the template preparation reaction by 50%.
	Library was mis-quantified.	Ensure that library was quantified accurately.
	Other.	Check the appropriate template preparation user guide for more information.
Low quality ISPs are present at high percentage (>15%)	Library input was too low.	Double the volume of library used in template preparation.
		Use a fresh dilution of library prepared in a low-bind tube.
	Other.	Check the appropriate template preparation user guide for more information.

Observation	Possible cause	Recommended action
Primer dimer is present during sequencing	Primer dimer formed during reaction set up.	Make sure to perform an additional round of 1X MagMAX™ Pure Bind Beads purification of the library (see "Purify the DNA library" on page 46 and "Purify the RNA library" on page 56).
		For challenging primer panels that produce excessive amounts of primer dimer, use the CRC reagent to your target amplification reaction to reduce the primer dimer product (see page 34 and 51).
		Amplify the targets using the alternative cycling option B as described in "Amplify the targets—DNA panel" on page 41.
		For significant primer-dimer content (~30%–70%), you can reduce the concentration of panel primer in target amplification reactions: • For panels with less than 500 primer pairs per pool, you can reduce the primer concentration by 5X, down to 10 nM. • For panels with 501–1,000 primer pairs per pool, you can reduce the primer concentration by 2.5X, down to 10 nM. • For panels with more than 1,000 primer pairs per pool, you can reduce the primer concentration down to 5 nM.

B

Supplemental procedures

Quantify the library by qPCR

Library concentration can be determined by qPCR with the Ion Library Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802). Libraries typically have yields of 1,000–40,000 pM. RNA libraries can have yields less than 500 pM. Yield is not indicative of library quality. 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.

IMPORTANT! We recommend that you quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument when you use a different sample type or a panel for the first time. For more information, see Chapter 8, "Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument".

Quantify library and calculate the dilution factor

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.

Standard	Control Library	Nuclease-free water[1]	Dilution factor	Concentration
1	5 µL undiluted Control Library	45 µL	1:10	6.8 pM
2	5 µL Std 1	45 µL	1:100	0.68 pM
3	5 µL Std 2	45 µL	1:1000	0.068 pM

[1] Not DEPC-treated

2. Dilute each sample library.
   a. Make a 1:100 dilution by combining 2 µL of library with 198 µL of nuclease-free water, vortex to mix, then centrifuge briefly to collect the contents.
   b. Add 2 µL of the 1:100 diluted library to 198 µL of nuclease-free water to make a 1:10,000 dilution, vortex to mix, then centrifuge briefly to collect the contents.

3. Prepare reaction mixtures. For each sample, control, and standard, combine the following components:

Component	Volume
2X Ion Library qPCR Master Mix	20 µL
Ion Library TaqMan™ Quantitation Assay, 20X	2 µL
Final volume	22 µL

4. Mix thoroughly, then pipet 11-µL aliquots into 2 wells of a 96-well PCR plate for each sample, control, and standard.

5. Add 9 µL of the diluted (1:10,000) Ion AmpliSeq™ HD library or 9 µL of each standard dilution to each well (two wells per sample as noted before), for a total reaction volume of 20 µL.

6. 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 instrument settings appropriate for your real-time PCR instrument, as listed in Table 6.

      The fast cycling program was developed using the StepOnePlus™ Real-Time PCR 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.

Table 6 Instrument settings

Real-time PCR System	Reaction plate	Run mode	Stage	Temperature	Time
7500 Fast	96-well Fast	Fast	Hold (UDG incubation)	50°C	2 minutes
7900 HT 7900 HT Fast	96-well Fast 384-well Standard		Hold (polymerase activation)	95°C	20 seconds
ViiA™ 7			Cycle (40 cycles)	95°C	1 second
QuantStudio™ Real-Time PCR Systems					
StepOne™ StepOnePlus™	48-/96-well Fast			60°C	20 seconds
7500	96-well Standard	Standard	Hold (UDG incubation)	50°C	2 minutes
			Hold (polymerase activation)	95°C	2 minutes
7900 HT 7900 HT Fast			Cycle (40 cycles)	95°C	15 second
ViiA™ 7				60°C	1 minutes
QuantStudio™ Real-Time PCR Systems					

7. Following qPCR, calculate the average concentration of the undiluted Ion AmpliSeq™ HD library by multiplying the concentration that is determined with qPCR by 10,000.

8. 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 dilution factor is 300 pM/100 pM = 3.
   - Therefore, 10 µL of library that is mixed with 20 µL of Low TE (1:3 dilution) yields approximately 100 pM.

9. Dilute the library to ~100 pM, then proceed to Chapter 9, “Combine libraries” or store libraries as described in “Store libraries”.

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.

Equalize the library

The Ion Library Equalizer™ Kit (Cat. No. 4482298) provides a method for normalizing library concentration at ~100 pM without the need for special instrumentation for quantification.

IMPORTANT!

- We recommend that you quantify libraries prepared using new Ion AmpliSeq™ HD panels or new sample types with the Agilent™ 2100 Bioanalyzer™ Instrument.
- The qPCR library quantification method is recommended for HD panels that have been determined to have low levels of primer dimers. When library yields are consistently above 0.5 nM, the Ion Library Equalizer™ Kit can be used reliably. If sample quality or quantity is variable or unknown (such as RNA from FFPE tissue or Direct FFPE DNA), the Agilent™ 2100 Bioanalyzer™ Instrument quantification method may provide a higher success rate in terms of library yield and the resulting number of sequencing reads.
- Standard library amplification parameters are not compatible with the Ion Library Equalizer™ Kit.

Ion Library Equalizer™ Kit

The Ion Library Equalizer™ Kit (Cat. No. 4482298) provides an optional, streamlined method for normalizing library concentration at 100 pM without quantification.

The kit contains reagents sufficient for 96 libraries.

Component	Amount	Storage
Equalizer™ Primers (pink cap)	200 µL	2°C to 8°C
Equalizer™ Capture (purple cap)	1 mL	
Equalizer™ Elution Buffer (clear cap)	10 mL	
Equalizer™ Beads (orange cap)	300 µL	
Equalizer™ Wash Buffer (clear cap)	35 mL	15–30°C[1]

[1] Can be stored at 2–8°C.

Before you begin

Warm all the reagents in the Ion Library Equalizer™ Kit and Platinum™ PCR SuperMix High Fidelity to room temperature. Vortex and centrifuge all reagents before use.

Amplify the library

1. Transfer 10 µL of the purified Ion AmpliSeq™ HD library to a new well of a 96-well plate, then add 50 µL of Platinum™ PCR SuperMix High Fidelity and 2 µL of Equalizer™ Primers to each library well. The Platinum™ PCR SuperMix High Fidelity and Equalizer™ Primers can be combined before addition.

   Do not use the Library Amplification Primer Mix from the Ion AmpliSeq™ Library Kit 2.0.

2. Seal the plate with a 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 compression pad on the plate, then load in the thermal cycler. Run the following program. During cycling, wash the Equalizer™ Beads, if they have not been previously washed.

Stage	Temperature	Time
Hold	98°C	2 minutes
8 cycles	98°C	15 seconds
	64°C	1 minute
Hold	10°C	Hold (up to 1 hour)

4. After thermal cycling, centrifuge the plate to collect any droplets.

5. *(Optional)* Confirm the concentration of the amplified library by removing 2 µL of the reaction and quantify using the Ion Library TaqMan™ Quantitation Kit (“Quantify the library by qPCR” on page 69) or the Agilent™ 2100 Bioanalyzer™ Instrument (Chapter 8, “Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument”).

   Use the Ion Library TaqMan™ Quantitation Kit only when the amplified library concentration is expected to exceed 4000 pM.

Wash the Equalizer™ Beads *(if not previously washed)*

1. Bring the Equalizer™ Beads to room temperature, then mix thoroughly.

   Beads for multiple reactions can be prepared in bulk and stored in Equalizer™ Wash Buffer at 4°C for up to 12 months until use. After 12 months, wash the beads again with an equal volume of Equalizer™ Wash Buffer.

2. For each reaction, pipet 3 µL of beads into a clean 1.5-mL tube, then add 6 µL per reaction of Equalizer™ Wash Buffer.

   For example, if you have 4 reactions, add 12 µL of beads and 24 µL of Equalizer™ Wash Buffer.

3. Place the tube in a magnetic rack for 3 minutes, or until the solution is clear.

4. Carefully remove the supernatant without disturbing the pellet, then discard.

5. Remove the tube from the magnet, add 6 µL per reaction of Equalizer™ Wash Buffer, then pipet up and down to resuspend.

Add Equalizer™ Capture to the amplified library

1. Carefully remove the seal from the plate, then add exactly 10 µL of Equalizer™ Capture reagent to each library amplification reaction.

   The final equalized library concentration depends on accurate pipetting of the Equalizer™ Capture reagent.

2. Seal the plate with a MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect the contents.

   Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

3. Incubate at room temperature for 5 minutes.

(Optional) Combine captured libraries

1. Determine the number of samples to be combined based on the number of libraries that can be run on a single chip. (see Chapter 9, “Combine libraries”).

2. Carefully remove the seal from the plate, then remove and combine an equal volume (10–30 µL) of each sample into a single well or tube. The volume of the combined library must be 60 µL or greater. Mix the combined libraries thoroughly, then transfer 60 µL to a new well. Treat the combined libraries as a single sample and proceed to the next section.

   Example: If 8 libraries are to be combined in a single templating and sequencing reaction, remove 10 µL of each library and combine them together in a new position on the 96-well plate.

   Note: Save uncombined individual libraries for repeat analysis, if needed.

Add Equalizer™ Beads and wash

1. Gently vortex or pipet up and down to mix the washed Equalizer™ Beads.

2. Carefully remove the seal from the plate, then add 6 µL of washed Equalizer™ Beads to each plate well thata contains the captured library.

3. Set the pipette volume to 40 µL, then pipet the mixture up and down at least 5 times to mix thoroughly.

4. Incubate at room temperature for 5 minutes.

   Check for droplets on the sides of the plate wells. If droplets are observed, seal the plate, then gently tap the plate on a hard, flat surface, or briefly centrifuge to collect the contents.

5. Place the plate in the magnet, then incubate for 2 minutes or until the solution is clear.

6. If needed, carefully remove the seal from the plate, then remove the supernatant without disturbing the pellet.

   Save the supernatant for repeat analysis, if needed.

7. Add 150 µL of Equalizer™ Wash Buffer to each reaction.

8. To wash the beads, move the plate side-to-side in the two positions of the magnet.

   If your magnet does not have two positions for shifting the beads, remove the plate from the magnet, set a pipettor to at least half the total volume, then gently pipet the contents up and down 5 times. Return the plate to the magnet and incubate for 2 minutes or until the solution clears.

9. With the plate still in the magnet, carefully remove, then discard the supernatant without disturbing the pellet.

10. Repeat the bead wash as described in step 7 through step 9.

    Ensure that as much wash buffer as possible is removed without disturbing the pellet.

Elute the equalized library

1. Remove the plate from the magnet, then add 100 µL of Equalizer™ Elution Buffer to each pellet.

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

   Centrifuge with enough force to collect the contents, but not the pellet beads. If beads are pelleted, vortex again and centrifuge more gently.

3. Elute the library by incubating in a thermal cycler at 32°C for 5 minutes.

4. Place the plate in the magnet, then incubate at room temperature for 5 minutes or until the solution is clear.

   The supernatant contains the equalized library at ~100 pM, which can be stored with beads for up to 1 month at 4–8°C.

Proceed to template preparation, or combine or store libraries as described in “Store libraries” on page 60.

(Optional) Combine equalized libraries

Combine the equalized libraries based on the number of libraries to be run on a single chip, as calculated in Chapter 9, “Combine libraries”.

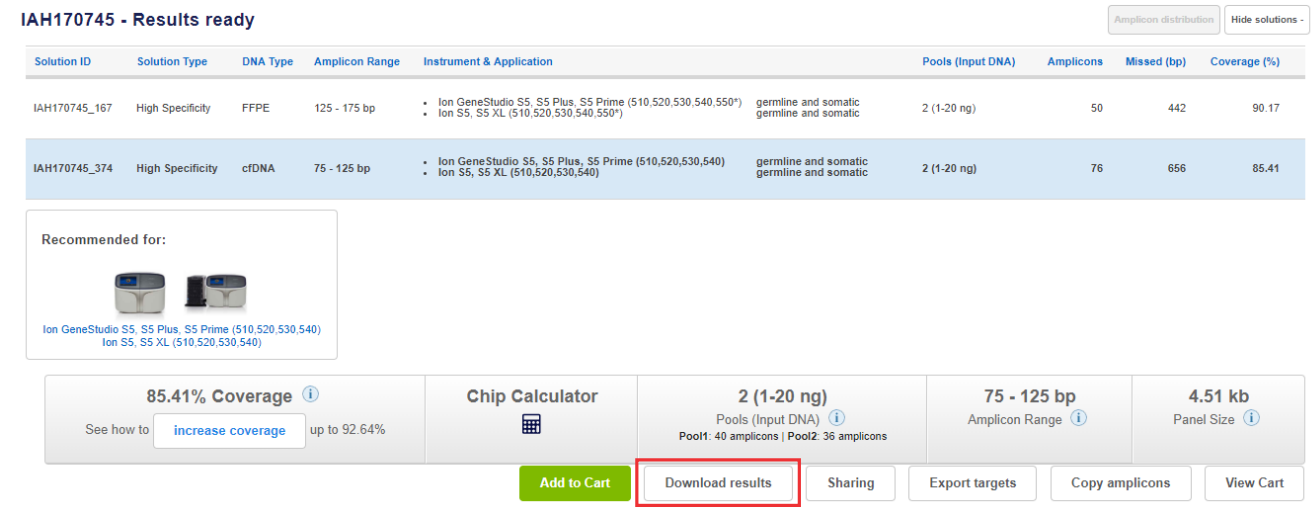
Store libraries

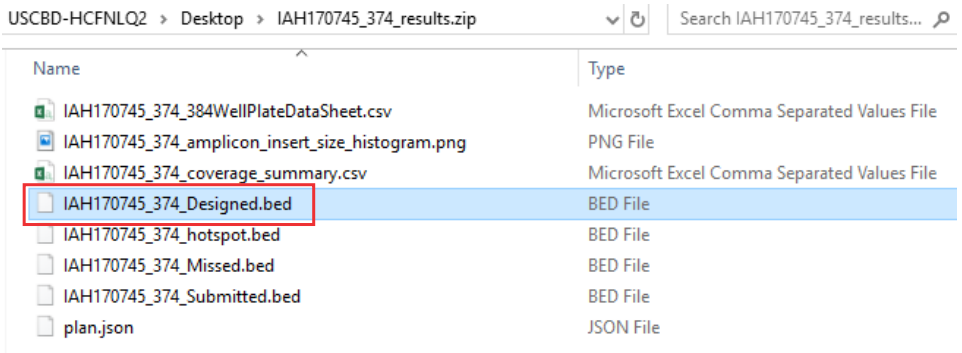
Store libraries at 4–8°C for up to 1 month. For longer lengths of time, store at –30°C to –10°C.

Download and install BED files

Download all available BED files specific for your Ion AmpliSeq™ HD panels from **AmpliSeq.com**, or contact your field service representative if you need help accessing the BED files.

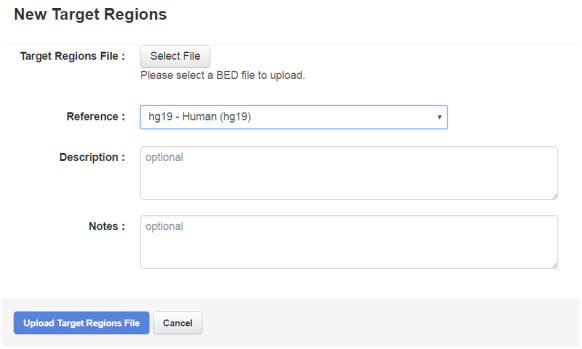
After you create the designs in Ion AmpliSeq™ Designer, download the results ZIP file. The results ZIP file contains the BED files for your designs.





1. Extract the BED file containing the ZIP file to a location of your choice.
2. Sign in to Torrent Suite™ Software where you want to install the **Target Regions** and **Hotspots** BED files.
3. Click the ⚙ **(Settings)** tab in the upper right of the screen, then select **Reference Sequences** from the dropdown list.
4. Upload the Target Regions panel BED file.
   a. In the left navigation menu, click **Target Regions**, then click **Add Target Regions**.
   b. From **Reference**, select **hg19 - Homo sapiens**.

c. Click **Select File**, then navigate to and select the **Target Regions** file specific for your panel.



d. Click **Open**, then click **Upload Target Regions File**.

5. Upload the Hotspots BED file.
   a. In the left navigation menu, click **Hotspots**, then click **Add Hotspots**.
   b. From **Reference**, select **hg19 - Homo sapiens**.
   c. Click **Select File**, then navigate to and select the Hotspots file specific for your panel.
   d. Click **Open**, then click **Upload Hotspots File**.

The **Target Regions** and **Hotspots** BED files EW upload to Torrent Suite™ Software and appear in the respective lists.

Create a custom workflow in Ion Reporter™ Software

Analysis workflow templates for Ion AmpliSeq™ HD

Ion Reporter™ Software 5.10 or later supports predefined analysis workflow templates for use with Ion AmpliSeq™ HD panels. The Ion AmpliSeq™ HD predefined analysis workflow templates must be activated by an administrator before use. To activate the templates, contact Technical Support or your support representative. After activation, identify the template that best matches your application, then use it to create a custom analysis workflow. For more information, see “Create a custom analysis workflow for use with Ion AmpliSeq™ HD panels” on page 79.

IMPORTANT! Ensure that the analysis workflow template name that you use matches the type of Ion AmpliSeq™ HD panel files that you include in your analysis. For example, if you use panel files for a liquid biopsy fusions single sample, copy the Ion AmpliSeq™ HD for Liquid Biopsy - w2.6 - Fusions - Single Sample analysis workflow template to create the custom analysis workflow.

Note: Workflows 2.6 are released with Ion Reporter™ Software 5.20.

Analysis workflow template	Description
Ion AmpliSeq™ HD for Tumor - w2.6 - DNA - Single Sample	Detects and annotates low frequency (to 0.5% limit of detection) variants (SNPs, INDELs and CNVs) from targeted DNA libraries using Ion AmpliSeq™ HD technology. Compatible with DNA that is purified from tumor samples.
Ion AmpliSeq™ HD for Tumor - w2.6 - Fusions - Single Sample	Detects and annotates gene fusions from targeted RNA libraries using Ion AmpliSeq™ HD technology. Compatible with RNA that is purified from tumor samples.
Ion AmpliSeq™ HD for Tumor - w2.6 - DNA and Fusions (Single Library) - Single Sample	Detects and annotates low frequency (to 0.5% limit of detection) variants (SNPs, INDELs, CNVs and fusions) from targeted nucleic acid libraries using Ion AmpliSeq™ HD technology. Compatible with DNA and RNA that is purified together from tumor samples.
Ion AmpliSeq™ HD for Tumor - w2.6 - DNA and Fusions (Separate Libraries) - Single Sample	Detects and annotates low frequency (to 0.5% limit of detection) somatic variants (SNPs, INDELs and CNVs) from targeted DNA libraries, as well as gene fusions from matching targeted RNA libraries using Ion AmpliSeq™ HD technology. Compatible with DNA and RNA that is purified separately from tumor samples.
Ion AmpliSeq™ HD for Liquid Biopsy - w2.6 - DNA - Single Sample	Detects and annotates low frequency (to 0.1% limit of detection) variants (SNPs, INDELs and CNVs) from targeted DNA libraries using Ion AmpliSeq™ HD technology. Compatible with DNA that is purified from cell-free liquid biopsy samples.
Ion AmpliSeq™ HD for Liquid Biopsy - w2.6 - Fusions - Single Sample	Detects and annotates gene fusions from targeted RNA libraries using Ion AmpliSeq™ HD technology. Compatible with RNA that is purified from cell-free liquid biopsy samples.
Ion AmpliSeq™ HD for Liquid Biopsy - w2.6 - DNA and Fusions (Single Library) - Single Sample	Detects and annotates low frequency (to 0.1% limit of detection) variants (SNPs, INDELs, CNVs and fusions) from targeted nucleic acid libraries using Ion AmpliSeq™ HD technology. Compatible with DNA and RNA that is purified together from cell-free liquid biopsy samples.
Ion AmpliSeq™ HD for Liquid Biopsy - w2.6 - DNA and Fusions (Separate Libraries) - Single Sample	Detects and annotates low frequency (to 0.1% limit of detection) somatic variants (SNPs, INDELs and CNVs) from targeted DNA libraries, and gene fusions from matching targeted RNA libraries using Ion AmpliSeq™ HD technology. Compatible with DNA and RNA that is purified separately from cell-free liquid biopsy samples.

Create a custom analysis workflow for use with Ion AmpliSeq™ HD panels

To analyze Ion AmpliSeq™ HD sequencing data in Ion Reporter™ Software, you must create a custom analysis workflow for use with your Ion AmpliSeq™ HD panels. To create a custom analysis workflow, first copy one of the preinstalled Ion AmpliSeq™ HD analysis workflow templates, then add target regions files, and any available hotspots files or CNV baseline. For RNA samples, you must also add fusion panel files to your custom analysis workflow.

For details on creating custom analysis workflows in Ion Reporter™ Software, see the Ion Reporter™ Software help system or the *Ion Reporter™ Software 5.20 User Guide* (Pub No. MAN0028322).

After you create the custom analysis workflow, you can select the analysis workflow in Torrent Suite™ Software to transfer data automatically to the appropriate Ion Reporter™ Server and use the analysis workflow for the data analysis in Ion Reporter™ Software.

1. Sign in to Ion Reporter™ Software.
2. In the **Workflows** tab, click **Overview**.

   For information on available Ion AmpliSeq™ HD analysis workflow templates, see “Analysis workflow templates for Ion AmpliSeq™ HD” on page 77.
3. In the **Workflows** table, select the Ion AmpliSeq™ HD analysis workflow template that you want to copy, then click **Actions** ▸ **Copy**.

   The **Edit** workflow bar opens to the **Research Application** step with the **Research Application** and **Sample Group** selected.

   When you copy analysis workflow templates, you cannot change these settings.

4. Click **Next**, then in the **Reference** step, select or upload the appropriate files.

Option	Description
Select a file that was previously uploaded to Ion Reporter™ Software.	Select the **Target Regions** and, optionally, **Hotspot Regions** files from the dropdown lists.
Upload the entire package of the Ion AmpliSeq™ HD panel files directly from **AmpliSeq.com**. When you use this option, a target regions file and any available hotspot regions or fusion files specific for your panel are uploaded.	Click **AmpliSeq Import**.
Import a target regions file that you previously downloaded from **AmpliSeq.com** and saved to your local storage.	a. Under the **Target Regions** list, click **Upload**. b. Click **Select file**, browse to, then select the target regions BED file, then click **Open**. c. Select **Ion AmpliSeq™ HD**, then click **Upload**.
(Optional) Upload a hotspot regions file that was previously uploaded from **AmpliSeq.com** and saved to your local storage.	a. Under the **Hotspots Regions** list, click **Upload**. b. Click **Select file**, browse to, then select the hotspot regions BED file, then click **Open**. c. Select **Ion AmpliSeq™ HD**, then click **Upload**.
If you use a panel that detects fusions, upload a FASTA file that was previously uploaded from **AmpliSeq.com** and saved to your local storage.	a. Under the **Fusion Panel** list, click **Upload**. b. Click **Select file**, browse to, then select the fusions ZIP file, then click **Open**. c. Select **Ion AmpliSeq™ HD**, then click **Upload**.

5. Click **Next**.

6. In the **Annotation** step, confirm or select an annotation set, confirm or select a MyVariants database, then click **Next**.

7. In the **Filters** step, select a filter chain from the **Filter Chains** list to change the default filter chain for analyses that use this analysis workflow. If you do not change the filter chain, the default filter chain is saved for the new analysis workflow.

Option	Description
Called Variants and Controls	This filter chain reports all variants (either hotspots or novel) that pass the filter and are not called as reference or NOCALL. Variant types include SNV, INDEL, MNV, CNV, LONGDEL, FUSION, EXPR_CONTROL, ASSAYS_5P_3P, RNA_HOTSPOT, GENE_EXPRESSION, RNAExonVariant, ProcControl, FLT3-ITD, and RNA Exon Tile.
Called Hotspot Variants and Controls	This filter chain reports all hotspot variants that pass the filter and are not called as reference or NOCALL. Variant types include SNV, INDEL, MNV, and RNA Exon Tile.
Variant Matrix Summary	Select this filter chain for analysis results that replicate data that is shown for Ion AmpliSeq™ HD analysis results in the **Variant Matrix Summary**. Variant types include: SNV/INDEL, CNV, fusions, and RNAExonVariants.
Oncomine™ Variants (5.10 or later)	Select this filter chain to show only the variants that are annotated with the Oncomine™ Variant Annotator plugin.

8. Click **Next**.

9. In the **Copy Number** step, select a copy number baseline from the **Baseline** list, if applicable, then click **Next**.

   IMPORTANT! To add a copy number baseline, contact your Field Bioinformatics Specialist (FBS).

10. In the **Plugins** step, select a plugin, then click **Next**.

    In the **Plugins** step, the Oncomine™ Variant Annotator plugin is selected by default. This plugin adds annotations for variants that are relevant to cancer with Oncomine™ Gene Class and Oncomine™ Variant Class information. This plugin is included by default with the Ion AmpliSeq™ HD analysis workflow templates. If you import the VCF file of analysis results into Oncomine™ Reporter, these annotations are included in a report that is generated from that software.

11. In the **Final Report** step, select or confirm the final report template that is selected in the list, then click **Next**.

12. In the **Parameters** step, make any required changes, then click **Next**.

    IMPORTANT! If you are using the Ion AmpliSeq™ HD test panel, consult your field support representative and other training materials before you change the parameters. If you designed a white glove panel, consult your white glove representative or field support representative to determine if parameter changes are required.

13. In the **Confirm** step, name the analysis workflow, enter an optional description, then click **Save Workflow**.

The newly created analysis workflow is added to the list of analysis workflows in the **Workflows** tab in the **Overview** screen.

C Prepare Ion AmpliSeq™ HD DNA libraries using CRC

This protocol is the legacy protocol without the use of 5X HD Enhancer. This protocol is designed for use with 1-, 2-, or 3-pool DNA primer panels.

Guidelines for DNA isolation, quantification, and input

- We recommend the MagMAX™ Cell-Free DNA Isolation Kit (Cat. No. A29319) for isolating cfDNA or MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit (Cat. No. A36716) to isolate cfNA.
- We recommend MagMAX™ FFPE DNA/RNA Ultra Kit (Cat. No. A31881), RecoverAll™ Multi-Sample RNA/DNA Workflow (Cat. No. A26069), or RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Cat. No. AM1975) to isolate DNA and RNA from formaldehyde- or paraformaldehyde-fixed paraffin-embedded (FFPE) tissue samples.
- We recommend that you use the Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851 or Q32854) to quantify cfDNA, cfNA, and FFPE DNA samples.
- Quantification methods such as densitometry (for example, using a NanoDrop™ Spectrophotometer) are not recommended because they are not specific for DNA. Use of these methods can lead to gross overestimation of the sample DNA concentration, under-seeding of the target amplification reaction, and low library yields.
- Each target amplification reaction per primer pool requires 20 ng of cfDNA or cfNA (≥1.2 ng/µL for 1 primer pool or ≥2.4 ng/µL for 2 primer pools) from human whole blood or bone marrow, or 20 ng of FFPE DNA (≥2.8 ng/µL) to achieve 0.1% limit of detection (LOD). For more information, see Appendix E, “Experimental design of a sequencing experiment using Ion AmpliSeq™ HD technology”.

Remove deaminated bases from FFPE DNA

IMPORTANT! If using DNA isolated from sources other than FFPE tissue, proceed directly to "Set up DNA molecular tagging reactions" on page 34.

FFPE preservation methods can lead to significant cytosine deamination of the isolated DNA, resulting in decreased sequencing quality. When using the Ion AmpliSeq™ HD Library Kit, deaminated cytosine (uracil) bases should be enzymatically removed by treatment with Uracil DNA Glycosylase (UDG) before the target amplification reaction.

1. For each FFPE DNA sample, add the following components to a single well of a 96-well PCR plate.

Component	Volume
20 ng FFPE DNA	≤7.3 µL
UDG, heat-labile	1 µL
Low TE	to 8.3 µL

2. Mix the reaction by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film. Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents.

   To prevent evaporation during UDG treatment, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

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

Temperature	Time
37°C	2 minutes
50°C	10 minutes
4°C	Hold (≤1 hour)

4. Remove the plate from the thermal cycler, then centrifuge briefly to collect the contents.

   STOPPING POINT Reactions can be stored at –20°C long term.

5. Carefully remove the plate seal, then proceed immediately to "Set up DNA molecular tagging reactions", adding the target amplification reaction components to the well containing 8.3 µL of UDG treated FFPE DNA.

Set up DNA target amplification reactions

Set up DNA target amplification reactions using one of the following procedures for either 1-, 2-, or 3-pool primer panels.

Prepare DNA target amplification reactions—1-pool primer panel

For 1-pool DNA primer panels (containing FWD and REV subpools), target amplification reactions can be assembled directly in a 96-well plate.

IMPORTANT!

- Keep all reagents on ice or in a prechilled 4°C cold block during reaction setup.
- 4X Ion AmpliSeq™ HD Amplification Mix is viscous. Pipet slowly and mix thoroughly.
- Do not combine FWD and REV primer subpools for storage. Primer subpools must remain separate and combined only during the target amplification reaction setup.

1. Place a 96-well plate on ice or in a prechilled 4°C cold block.

2. Add the following components to a single well of a 96-well PCR plate. Prepare a master mix without sample DNA for multiple reactions.

   The CRC reagent improves target amplification reactions for challenging panels that produce excessive amounts of primer dimer or panels that have 500 or more primer pairs. We recommend that you use the CRC reagent with all panel types.

Component	Volume
20 ng cfNA, cfDNA, or FFPE DNA	≤12.5 µL
4X Ion AmpliSeq™ HD Amplification Mix (purple cap)	7.5 µL
10X Pool 1 FWD (red cap)	3 µL
10X Pool 1 REV (yellow cap)	3 µL
CRC (yellow cap)	4 µL
Nuclease-free water	to 30 µL

3. Mix by pipetting at least half the total volume up and down at least 5 times, then seal the plate with a MicroAmp™ Clear Adhesive Film. Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

Proceed to “Amplify the targets” on page 88.

Prepare DNA target amplification reactions—2-pool primer panel

If you are using a 2-pool DNA primer panel (containing 2 FWD subpools and 2 REV subpools), set up two 15-µL amplification reactions, then combine them after target amplification to give a total volume of 30 µL.

IMPORTANT!

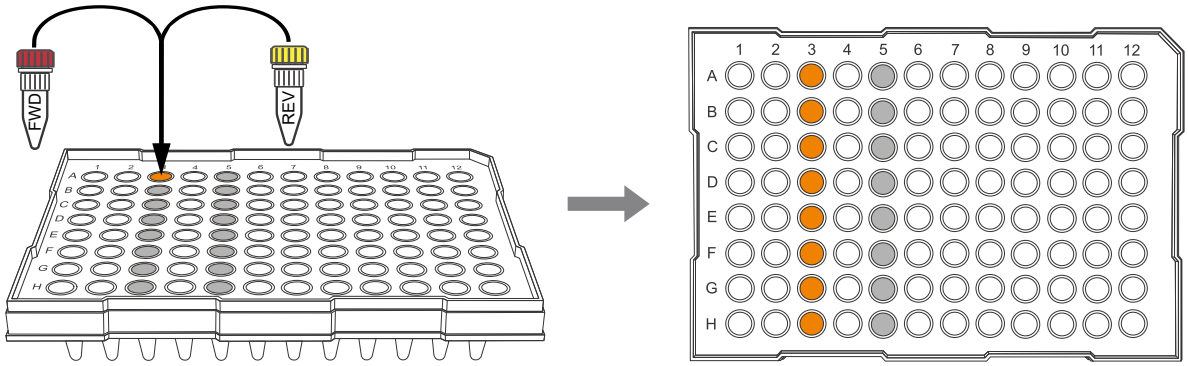
- Keep all reagents on ice or in a prechilled 4°C cold block during reaction setup.
- 4X Ion AmpliSeq™ HD Amplification Mix is viscous. Pipet slowly and mix thoroughly.
- Do not combine FWD and REV primer subpools together for storage. Primer subpools must remain separate and combined only during the target amplification reaction setup.

1. Place a 96-well plate on ice or in a prechilled 4°C cold block.

2. Add the following components to two wells of a 96-well PCR plate. For multiple reactions, prepare a master mix without the sample DNA.

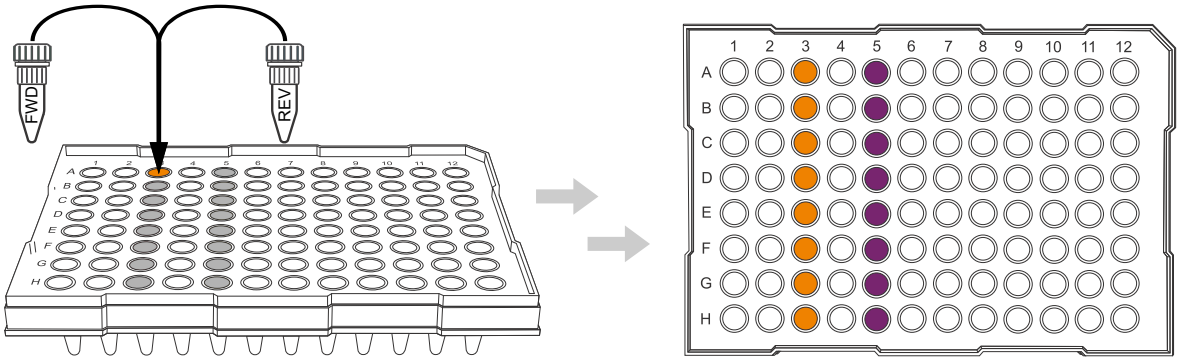
   The CRC reagent improves target amplification reactions for challenging panels that produce excessive amounts of primer dimer or panels that have 500 or more primer pairs. We recommend that you use the CRC reagent with all panel types.

Component	Well 1, Pool 1 (volume)	Well 2, Pool 2 (volume)
20 ng cfNA, cfDNA, or FFPE DNA	≤6.3 µL	≤6.3 µL
4X Ion AmpliSeq™ HD Amplification Mix (purple cap)	3.7 µL	3.7 µL
10X Primer Pool 1 FWD (red cap)	1.5 µL	–
10X Primer Pool 1 REV (yellow cap)	1.5 µL	–
10X Primer Pool 2 FWD (blue cap)	–	1.5 µL
10X Primer Pool 2 REV (green cap)	–	1.5 µL
CRC (yellow cap)	2 µL	2 µL
Nuclease-free water	to 15 µL	to 15 µL



Wells that contain DNA, 4X Ion AmpliSeq™ HD Amplification Mix, and CRC only

Wells that have Pool 1 FWD and Pool 1 REV added



- Wells that contain DNA, 4X Ion AmpliSeq™ HD Amplification Mix, and CRC only
- Wells that have Pool 1 FWD and Pool 1 REV added
- Wells that have Pool 2 FWD and Pool 2 REV added

3. Mix thoroughly by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film. Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

Proceed to “Amplify the targets” on page 88.

Prepare DNA target amplification reactions—3-pool primer panel

If you are using a 3-pool DNA primer panel (containing 3 FWD subpools and 3 REV subpools), set up three 10-µL amplification reactions, then combine them after target amplification to give a total volume of 30 µL.

IMPORTANT!

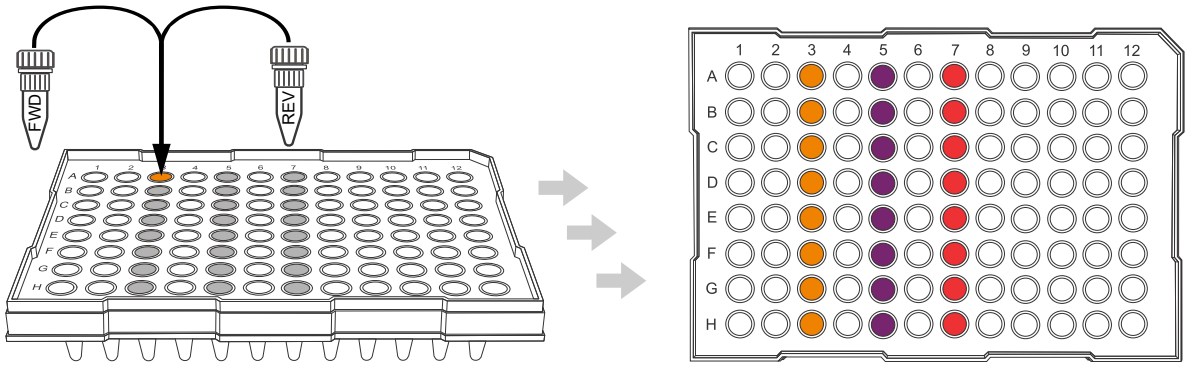
- Keep all reagents on ice or in a prechilled 4°C cold block during reaction setup.
- 4X Ion AmpliSeq™ HD Amplification Mix is viscous. Pipet slowly and mix thoroughly.
- Do not combine FWD and REV primer subpools together for storage. Primer subpools must remain separate and combined only during the target amplification reaction setup.

1. Place a 96-well plate on ice or in a prechilled 4°C cold block.

2. Add the following components to three wells of a 96-well PCR plate. For multiple reactions, prepare a master mix without the sample DNA.

   The CRC reagent improves target amplification reactions for challenging panels that produce excessive amounts of primer dimer or panels that have 500 or more primer pairs. We recommend that you use the CRC reagent with all panel types.

Component	Well 1, Pool 1 (volume)	Well 2, Pool 2 (volume)	Well 3, Pool 3 (volume)
20 ng cfNA, cfDNA, or FFPE DNA	≤4.2 µL	≤4.2 µL	≤4.2 µL
4X Ion AmpliSeq™ HD Amplification Mix (purple cap)	2.5 µL	2.5 µL	2.5 µL
10X Primer Pool 1 FWD (red cap)	1 µL	–	–
10X Primer Pool 1 REV (yellow cap)	1 µL	–	–
10X Primer Pool 2 FWD (blue cap)	–	1 µL	–
10X Primer Pool 2 REV (green cap)	–	1 µL	–
10X Primer Pool 3 FWD (pink cap)	–	–	1 µL
10X Primer Pool 3 REV (white cap)	–	–	1 µL
CRC (yellow cap)	1.3 µL	1.3 µL	1.3 µL
Nuclease-free water	to 10 µL	to 10 µL	to 10 µL



- Wells that contain DNA, 4X Ion AmpliSeq™ HD Amplification Mix, and CRC only
- Wells that have Pool 1 FWD and Pool 1 REV added
- Wells that have Pool 2 FWD and Pool 2 REV added
- Wells that have Pool 3 FWD and Pool 3 REV added

3. Mix thoroughly by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film. Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and the plate perimeter.

Proceed to “Amplify the targets” on page 88.

Amplify the targets

Choose the best cycling program to amplify the targets based on the type of panel that is used.

IMPORTANT! If you are using challenging primer panels or panels that have 500 or more primer pairs, use cycling option A to amplify the targets.

1. Place a MicroAmp™ Optical Film Compression Pad on the plate, then load the plate into the thermal cycler.
2. Run one of the following cycling programs, depending on the type of panel that is used.
   - *(Recommended)* Use the cycling option A program for all primer panel types, including challenging panels or panels that have 500 or more primer pairs.

     Table 7 Cycling option A

Stage	Step	Temperature	Time
3 cycles	Denature	99°C	30 seconds
	Anneal	64°C	2 minutes
		60°C	12 minutes
		66°C	2 minutes
	Extend	72°C	2 minutes
Hold	Final extension	72°C	2 minutes
Hold	—	4°C	≤1 hour

   - Use the cycling option B alternative cycling program if excessive amounts of primer dimer is produced with cycling option A.

     Table 8 Cycling option B

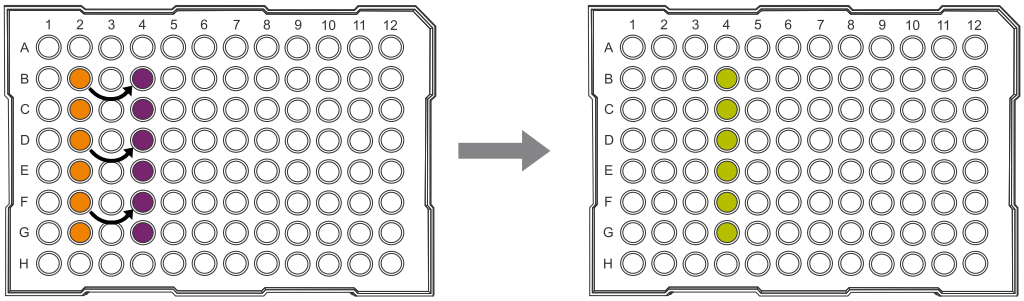
Stage	Step	Temperature	Time
3 cycles	Denature	99°C	30 seconds
	Anneal	64°C	2 minutes
		60°C	6 minutes
	Extend	72°C	30 seconds
Hold	Final extension	72°C	2 minutes
Hold	—	4°C	≤1 hour

Combine target amplification reactions (for 2-pool DNA primer panel libraries)

Note: Combining target amplification reactions is required only for DNA libraries with 2 or more primer pools.

For instructions on combining target amplification reactions for 3-pool DNA primer panel libraries, see "Prepare molecular tagging reactions—3-pool primer panel" on page 38.

1. Remove the plate from the thermal cycler, then centrifuge briefly to collect the contents.
2. Carefully remove the plate seal.
3. For each sample, combine both 15-µL target amplification reactions into a single well.



- Wells with Pool 1 FWD and Pool 1 REV target amplification reactions
- Wells with Pool 2 FWD and Pool 2 REV target amplification reactions
- Wells with combined Primer Pool 1 and Primer Pool 2 target amplification reactions

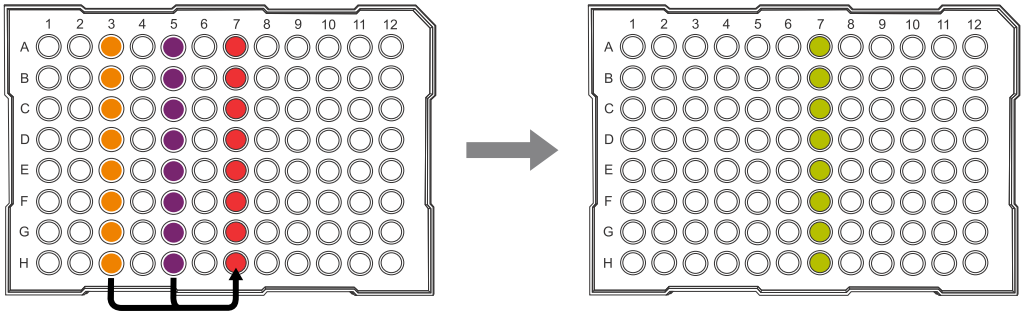
The total volume for each sample should be 30 µL.

Proceed to "Partially digest amplicons" on page 90.

Combine target amplification reactions (for 3-pool DNA primer panel libraries)

Note: Combining target amplification reactions is required only for DNA libraries with 2 or more primer pools.

1. Remove the plate from the thermal cycler, then centrifuge briefly to collect the contents.
2. Carefully remove the plate seal.
3. For each sample, combine all three 10-µL target amplification reactions into a single well for a total volume of 30 µL per sample.



- Wells with Pool 1 FWD and Pool 1 REV target amplification reactions
- Wells with Pool 2 FWD and Pool 2 REV target amplification reactions
- Wells with Pool 3 FWD and Pool 3 REV target amplification reactions
- Wells with combined Primer Pool 1, Primer Pool 2, and Primer Pool 3 target amplification reactions

Proceed to “Partially digest amplicons” on page 90.

Partially digest amplicons

IMPORTANT!
- Ion AmpliSeq™ HD SUPA Reagent is viscous. Pipet slowly and mix thoroughly. Perform this step on ice or a cold block, then quickly proceed to incubation.
- Do not substitute any assay components with reagents from other kits.

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 5 µL of Ion AmpliSeq™ HD SUPA Reagent** (green cap) to each well.

   IMPORTANT! FuPa Reagent is NOT a substitute for the Ion AmpliSeq™ HD SUPA Reagent.

3. Mix by pipetting at least half the total volume up and down at least 5 times, then seal the plate with a MicroAmp™ Clear Adhesive Film.

   Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents. Avoid generating excessive bubbles.

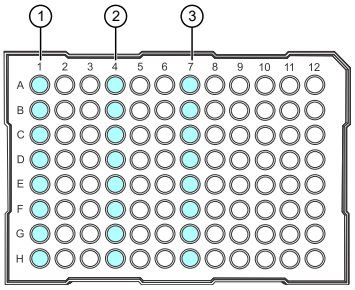
4. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate into the thermal cycler, then run the following program.

Temperature	Time
30°C	15 minutes
50°C	15 minutes
55°C	15 minutes
25°C	10 minutes
98°C	2 minutes
4°C	Hold (≤1 hour)

Amplify the library with barcoded primers

Ion AmpliSeq™ HD Dual Barcode Kit 1–24 primers are provided ready-to-use with different barcode primers per well. These primers are required for library preparation when sequencing one or multiple libraries per sequencing chip. If you are sequencing multiple libraries on a single chip, ensure that you are using different primers with each library.

Note: A blue dye is added to the barcode primers to help identify wells that contain primers. Columns 1, 4, and 7 in a 96-well plate contain primers in liquid format. Briefly centrifuge the plate before use.



① Column 1 contains barcode primers 1–8 in ascending order from top to bottom.
② Column 4 contains barcode primers 9–16 in ascending order from top to bottom.
③ Column 7 contains barcode primers 17–24 in ascending order from top to bottom.

IMPORTANT! When preparing libraries, we recommend that you alternate barcodes between consecutive sequencing runs to prevent carryover contamination. For more information, see “Procedural guidelines” on page 19.

1. Remove the plate from the thermal cycler, then centrifuge briefly to collect the contents.
2. Carefully remove the adhesive film from the plate.
3. **Add 4 µL of the selected barcode primers from the Ion AmpliSeq™ HD Dual Barcode Kit 1–24** to each well.
4. Mix by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film.

   Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents.
5. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate into the thermal cycler, then run the following program.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	15 seconds
5 cycles	Denature	99°C	15 seconds
	Anneal	62°C	20 seconds
	Extend	72°C	20 seconds
12–15 cycles[1], see Table 9.	Denature	99°C	15 seconds
	Extend	70°C	40 seconds
Hold	Final extension	72°C	5 minutes
Hold	—	4°C	Indefinite

[1] For FFPE DNA, using 17 cycles can improve library yields.

Table 9 Recommended number of amplification cycles

Primer pairs per panel	Number of cycles
12–500	15
501–1,000	14
1,001–2,000	13
2,001–5,000	12

STOPPING POINT Library amplification products can be stored at 4°C overnight on the thermal cycler. For longer periods, store at –20°C.

Purify the DNA 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.

Before you begin:

- Prepare 70% ethanol (650 µL × # of samples) fresh daily.
- If the library reaction plate was frozen, thaw the plate at room temperature.
- Briefly centrifuge the plate to collect the contents.

1. Carefully remove the plate seal, transfer each library into a separate new well, then add 10 µL of Low TE to each library well. Mix by pipetting at least half the total volume up and down 5 times.
2. Add 39 µL (1X sample volume before Low TE addition) of the MagMAX™ Pure Bind Beads to each library, then pipet up and down 5 times to thoroughly mix the bead suspension with the DNA. Avoid generating excessive bubbles.
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 is clear.
5. Carefully remove, then discard the supernatant without disturbing the pellet.
6. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet 5 times to wash the beads.
7. Carefully remove, then discard the supernatant without disturbing the pellet.
8. Repeat step 6 and step 7 one more time.
9. 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.
10. Remove the plate from the magnet, add 50 µL of Low TE buffer to each well, then resuspend the MagMAX™ Pure Bind Beads by pipetting up and down to disperse the beads into the solution.
11. Incubate the plate at room temperature for 5 minutes.
12. Place the plate back in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution clears.

13. Transfer 50 µL of the purified library into a new well.

14. Add 50 µL (1X sample volume) of MagMAX™ Pure Bind Beads to each library, then pipet up and down 5 times to thoroughly mix the bead suspension with the DNA.

    Avoid generating excessive bubbles. Visually inspect each well to ensure that the mixture is homogeneous.

15. Incubate the mixture for 5 minutes at room temperature.

16. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution is clear.

17. Carefully remove, then discard the supernatant without disturbing the pellet.

18. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet 5 times to wash the beads.

19. Carefully remove, then discard the supernatant without disturbing the pellet.

20. Repeat step 18 and step 19 one more time.

21. 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.

22. Remove the plate from the magnet, add 50 µL of Low TE buffer to each well, then resuspend the MagMAX™ Pure Bind Beads by pipetting up and down.

23. Incubate the plate at room temperature for 5 minutes.

24. Place the plate back in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution is clear.

25. Transfer the cleared library solution into a new tube or well in a plate.

Library quantification

Quantify the DNA library using one of the following options.

- Option 1—Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument (see Chapter 8, “Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument”).

  IMPORTANT! We recommend that you quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument when you use a different sample type or a panel for the first time.

- Option 2—Quantify the library by qPCR (see “Quantify the library by qPCR” on page 69).

Prepare Ion AmpliSeq™ HD RNA libraries without using 5X HD Enhancer

This protocol is the legacy protocol without the use of 5X HD Enhancer. This protocol is designed for use with 1-pool RNA primer panels and is not compatible for use with panels that contain both DNA and RNA primers.

Guidelines for RNA isolation, quantification, and input

- We recommend that you use the MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit (Cat. No. A36716) to isolate cfNA.
- We recommend that you use the MagMAX™ FFPE DNA/RNA Ultra Kit (Cat. No. A31881) or RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Cat. No. AM1975) for sequential isolation of DNA and RNA from the same formaldehyde- or paraformaldehyde-fixed paraffin-embedded (FFPE) tissue samples.
- We recommend that you use the Qubit™ RNA HS Assay Kit (Cat. No. Q32852) to quantify RNA.
- Each reverse transcription reaction requires 20 ng of cfNA or FFPE RNA (≥2.8 ng/µL).
- In general, library yield from high quality RNA is greater than from degraded samples. Library yield is not indicative of sequencing performance.
- If the library yield is below 100 pM, you can still proceed to sequencing by using a proportionally larger volume of the library when combining libraries or during template preparation.

Reverse transcribe the RNA

For this legacy RNA protocol, use the SuperScript™ VILO™ cDNA Synthesis Kit (Cat. No. 11754050) instead of the Ion Torrent™ NGS Reverse Transcription Kit that is used in the current protocol.

IMPORTANT! Warm the 5X VILO™ Reaction Mix at room temperature for at least 20 minutes, then vortex to mix before pipetting. If there is any visible precipitate, vortex to mix further until the 5X VILO™ Reaction Mix is completely resuspended.

1. If the RNA was prepared from FFPE tissue and not previously heat-treated, heat at 80°C for 10 minutes, then cool to room temperature.

2. For each sample, add the following components to a single well of a 96-well PCR plate. For multiple reactions, prepare a master mix without sample RNA.

Component	Volume
5X VILO™ Reaction Mix	2.4 µL
10X SuperScript™ Enzyme Mix	1.2 µL
≥20 ng cfNA or FFPE RNA	≤8.4 µL
Nuclease-free water	to 12 µL

3. Mix by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film. Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents.

   To prevent evaporation during the reverse transcription reaction, use an applicator tool to press the film securely around each reaction well and around the perimeter of 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.

Temperature	Time
42°C	30 minutes
85°C	5 minutes
4°C	Hold

STOPPING POINT Samples can be stored at 4°C for up to 16 hours in the thermal cycler. For longer term, store at –20°C.

5. Gently tap the plate on the bench to ensure that reactions are at the bottom of the wells. Alternatively, centrifuge the plate to collect any droplets.

Prepare cDNA target amplification reactions

IMPORTANT!

- Keep all reagents on ice or in a pre-chilled 4°C cold block during reaction setup.
- 4X Ion AmpliSeq™ HD Amplification Mix is viscous. Pipet slowly and mix thoroughly.
- Do not combine FWD and REV primer subpools for storage. Primer subpools must remain separate until combined during the target amplification reaction setup.
- **DO NOT** use CRC in RNA library preparation. The reagent is not compatible with the VILO™ Reaction Mix.

1. Place a 96-well plate on ice or in a prechilled 4°C cold block.

2. Add the following components to a single well of a 96-well PCR plate. Prepare a master mix without sample cDNA for multiple reactions.

Component	Volume
cDNA from 20 ng cfNA or FFPE RNA	12 µL[1]
4X Ion AmpliSeq™ HD Amplification Mix (purple cap)	7.5 µL
10X Pool 1 FWD (red cap)	3 µL
10X Pool 1 REV (yellow cap)	3 µL
Nuclease-free water	to 30 µL

[1] cDNA volume in each target amplification reaction is the entire volume of the reverse transcription reaction.

3. Mix by pipetting at least half the total volume up and down at least 5 times, then seal the plate with a MicroAmp™ Clear Adhesive Film. Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents.

   To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

Amplify the targets

Choose the best cycling program to amplify the targets based on the type of panel that is used.

IMPORTANT! If you are using challenging primer panels or panels that have 500 or more primer pairs, use cycling option A to amplify the targets.

1. Place a MicroAmp™ Optical Film Compression Pad on the plate, then load the plate into the thermal cycler.
2. Run one of the following cycling programs, depending on the type of panel that is used.
   - *(Recommended)* Use the cycling option A program for all primer panel types, including challenging panels or panels that have 500 or more primer pairs.

     Table 10 Cycling option A

Stage	Step	Temperature	Time
3 cycles	Denature	99°C	30 seconds
	Anneal	64°C	2 minutes
		60°C	12 minutes
		66°C	2 minutes
	Extend	72°C	2 minutes
Hold	Final extension	72°C	2 minutes
Hold	—	4°C	≤1 hour

   - Use the cycling option B alternative cycling program if excessive amounts of primer dimer is produced with cycling option A.

     Table 11 Cycling option B

Stage	Step	Temperature	Time
3 cycles	Denature	99°C	30 seconds
	Anneal	64°C	2 minutes
		60°C	6 minutes
	Extend	72°C	30 seconds
Hold	Final extension	72°C	2 minutes
Hold	—	4°C	≤1 hour

Partially digest amplicons

IMPORTANT!

- Ion AmpliSeq™ HD SUPA Reagent is viscous. Pipet slowly and mix thoroughly. Perform this step on ice or a cold block, then quickly proceed to incubation.
- Do not substitute any assay components with reagents from other kits.

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 5 µL of Ion AmpliSeq™ HD SUPA Reagent** (green cap) to each well.

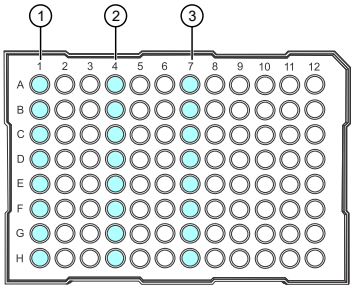
   IMPORTANT! FuPa Reagent is NOT a substitute for the Ion AmpliSeq™ HD SUPA Reagent.
3. Mix by pipetting at least half the total volume up and down at least 5 times, then seal the plate with a MicroAmp™ Clear Adhesive Film. Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents. Avoid generating excessive bubbles.
4. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate into the thermal cycler, then run the following program.

Temperature	Time
30°C	15 minutes
50°C	15 minutes
55°C	15 minutes
25°C	10 minutes
98°C	2 minutes
4°C	Hold (≤1 hour)

Amplify the library with barcoded primers

Ion AmpliSeq™ HD Dual Barcode Kit 1–24 primers are provided ready-to-use with different barcode primers per well. These primers are required for library preparation when sequencing one or multiple libraries per sequencing chip. If you are sequencing multiple libraries on a single chip, ensure that you are using different primers with each library.

Note: A blue dye is added to the barcode primers to help identify wells that contain primers. Columns 1, 4, and 7 in a 96-well plate contain primers in liquid format. Briefly centrifuge the plate before use.



① Column 1 contains barcode primers 1–8 in ascending order from top to bottom.
② Column 4 contains barcode primers 9–16 in ascending order from top to bottom.
③ Column 7 contains barcode primers 17–24 in ascending order from top to bottom.

IMPORTANT! When preparing libraries, we recommend that you alternate barcodes between consecutive sequencing runs to prevent carryover contamination. For more information, see "Procedural guidelines" on page 19.

1. Remove the plate from the thermal cycler, then centrifuge briefly to collect the contents.
2. Carefully remove the adhesive film from the plate.
3. Add 4 µL of the selected barcode primers from the Ion AmpliSeq™ HD Dual Barcode Kit 1–24 to each well.
4. Mix by pipetting at least half the total volume up and down at least 5 times, then seal the plate with MicroAmp™ Clear Adhesive Film. Alternatively, seal the plate, vortex for 5 seconds to mix the reactions, then centrifuge briefly to collect the contents.
5. Place a MicroAmp™ Optical Film Compression Pad on the plate, load the plate into the thermal cycler, then run the following program.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	15 seconds
5 cycles	Denature	99°C	15 seconds
	Anneal	62°C	20 seconds
	Extend	72°C	20 seconds
12–17 cycles	Denature	99°C	15 seconds
	Extend	70°C	40 seconds
Hold	Final extension	72°C	5 minutes
Hold	—	4°C	Indefinite

STOPPING POINT Library amplification products can be stored at 4°C overnight on the thermal cycler. For longer periods, store at –20°C.

Purify the RNA 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.

Before you begin:

- Prepare 70% ethanol (650 µL × # of samples) fresh daily.
- If the library reaction plate was frozen, thaw the plate at room temperature.
- Briefly centrifuge the plate to collect the contents.

1. Carefully remove the plate seal, transfer each library into a separate new well, then add 10 µL of Low TE to each library well. Mix by pipetting at least half the total volume up and down 5 times.
2. Add 39 µL (1X sample volume before Low TE addition) of the MagMAX™ Pure Bind Beads to each library, then pipet up and down 5 times to thoroughly mix the bead suspension with the DNA. Avoid generating excessive bubbles.
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 is clear.
5. Carefully remove, then discard the supernatant without disturbing the pellet.
6. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet 5 times to wash the beads.
7. Carefully remove, then discard the supernatant without disturbing the pellet.
8. Repeat step 6 and step 7 one more time.
9. 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.
10. Remove the plate from the magnet, add 50 µL of Low TE buffer to each well, then resuspend the MagMAX™ Pure Bind Beads by pipetting up and down to disperse the beads into the solution.
11. Incubate the plate at room temperature for 5 minutes.
12. Place the plate back in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution clears.

13. Transfer 50 µL of the purified library into a new well.

14. Add 50 µL (1X sample volume) of MagMAX™ Pure Bind Beads to each library, then pipet up and down 5 times to thoroughly mix the bead suspension with the DNA.

    Avoid generating excessive bubbles. Visually inspect each well to ensure that the mixture is homogeneous.

15. Incubate the mixture for 5 minutes at room temperature.

16. Place the plate in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution is clear.

17. Carefully remove, then discard the supernatant without disturbing the pellet.

18. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet 5 times to wash the beads.

19. Carefully remove, then discard the supernatant without disturbing the pellet.

20. Repeat step 18 and step 19 one more time.

21. 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.

22. Remove the plate from the magnet, add 50 µL of Low TE buffer to each well, then resuspend the MagMAX™ Pure Bind Beads by pipetting up and down.

23. Incubate the plate at room temperature for 5 minutes.

24. Place the plate back in a magnetic rack such as the DynaMag™–96 Side Magnet, then incubate for 2 minutes or until the solution is clear.

25. Transfer the cleared library solution into a new tube or well in a plate.

Library quantification

Quantify the RNA library using one of the following options.

- Option 1—Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument (see Chapter 8, “Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument”).

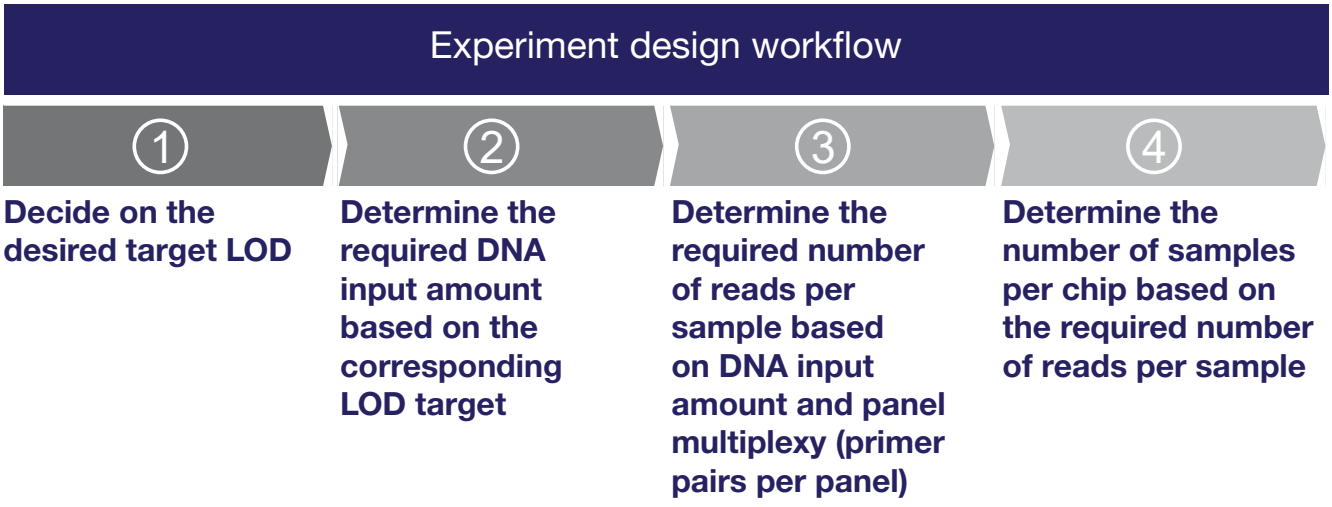
  IMPORTANT! We recommend quantifying the library with Agilent™ 2100 Bioanalyzer™ Instrument when you use a different sample type or a panel for the first time.

- Option 2—Quantify the library by qPCR (see “Quantify the library by qPCR” on page 69).

E

Experimental design of a sequencing experiment using Ion AmpliSeq™ HD technology

Ion AmpliSeq™ HD sequencing experiment design



Molecular tag sequencing technology—Important factors

When designing a sequencing experiment, using either Ion AmpliSeq™ (non-molecular tag) or Ion AmpliSeq™ HD (molecular tag-based) sequencing, three factors must be taken into consideration: the targeted % variant allele frequency limit of detection (%VAF LOD), the quantity of input DNA, and the minimum read depth needed.

To detect a variant in a DNA sample, the physical presence of multiple copies of that variant containing molecule (nominally 6–9 copies) is needed to reliably capture target variant molecules for variant calling. Assuming about 300 molecules per ng of human DNA, theoretically to detect variants at 1% frequency, 2-3 ng human DNA sample input is needed to get the 6-9 variant containing molecules in the assay. For 0.1% VAF LOD detection, 20-30 ng human DNA sample input is required to get the 6-9 variant containing molecules. The direct tie between DNA sample input amount requirement and the %VAF

LOD target in an assay applies for both Ion AmpliSeq™ (non-molecular tag) and Ion AmpliSeq™ HD (molecular tag-based) sequencing.

For assays that use Ion AmpliSeq™ chemistry, the required minimum DNA input amount and the minimum read depth are independently tied to the %VAF LOD target and the minimum reads depth requirement is not directly dependent on the DNA input amount but on the level of %VAF LOD target. Therefore, the lower the %VAF LOD target, the higher the required minimum read depth needed. To detect 1% variant allele frequency, 4,000X read depth is needed to get the 40 variant containing reads for variant calling.

In contrast, Ion AmpliSeq™ HD assays use molecular tag sequencing technology to achieve lower %VAF LOD targets. This relies on generating families of reads based on molecular tagging information before calling variants. So, the minimum reads depth requirement is directly dependent on the DNA input amount. That is, with increased DNA input, you must increase the minimum reads depth accordingly. This direct tie between DNA input amount and minimum reads depth needed is because of the requirement to build molecular tag families for variant calling. Therefore, increasing the DNA input amount and the concomitant increase in read depth results in low % VAF LOD detection (Figure 2).

In general, to build valid tag families at a given DNA input amount, the goal is to generate an average of 8 reads per amplicon for each input molecule (by default the minimum is 3 reads, with at least 1 read from each strand). For example, a human DNA sample with 20 ng input (~6,000 molecules) typically generates ~6,000 tag families per amplicon and can therefore theoretically achieve 0.1% LOD (6 / 6,000), when coverage depth is ≥50,000X (corresponding to an average of 8 reads per molecule per amplicon (8 × 6,000)). If the minimum coverage depth for a given input is not achieved, valid molecular tag families can be underrepresented, impairing the variant calling process. This also applies to DNA input amounts <20 ng. The reduction in the number of molecules in a sample results in the reduction of the number of tag families for higher %AF LOD targets, and consequently, a reduction of the reads depth requirement.

For 0.1%VAF LOD target with Ion AmpliSeq™ HD chemistry, we recommend at least 20–30 ng DNA input (50 ng for optimal performance). An optimal read coverage of 70,000X read depth per amplicon, which means 70,000 × (number of amplicons in the DNA panel) reads per library is recommended.

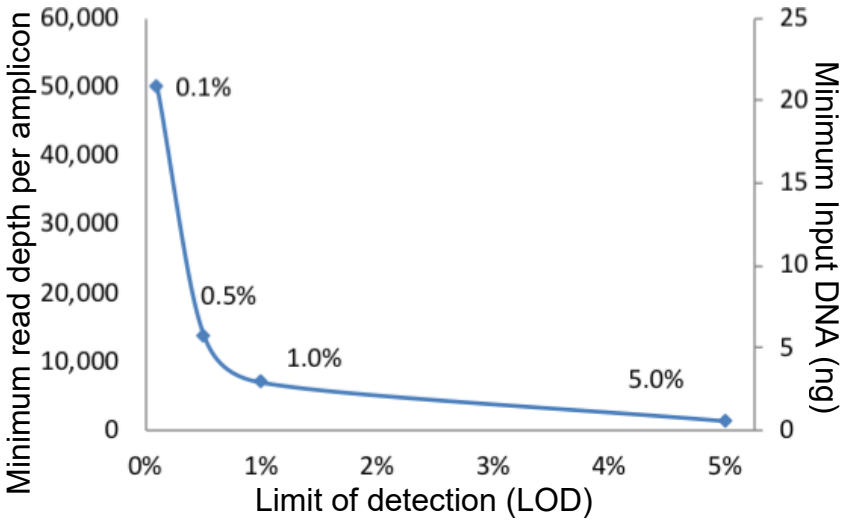


Figure 2 Representative limit of detection for a custom Ion AmpliSeq™ HD panel, using human cfDNA input and the required per amplicon read depth.

The graph illustrates the theoretical relationship between the minimum human DNA input amount, and the minimum reads depth per amplicon requirement to achieve a given %VAF LOD target. For example, to achieve 1%VAF LOD target, you need 2–3 ng DNA input and 7,000 reads depth per amplicon assuming the sample contains 6–9 copies of variant containing molecules and the read depth generates sufficient tag families for variant calling. If you increase the DNA input amount, you must increase the minimum read depth accordingly even you are still targeting the same %VAF LOD. The read depth increase is to resolve the molecular tag families due to the increased number of DNA input molecules.

The number of samples that can be sequenced on a single chip is determined by the total reads needed for a given assay and the number of reads that are generated per chip. Because the total reads requirement increases with decreasing target %VAF, fewer samples can be sequenced per chip. By raising the target %VAF you can start with a lower DNA input amount, concomitantly reduce the needed total read depth, and therefore sequence a greater number of samples per chip.

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

Documentation and support

Related documentation

Document	Publication number
Ion AmpliSeq™ HD Library Kit Quick Reference	MAN0017774
Ion Reporter™ Software 5.20 User Guide	MAN0028321
Torrent Suite™ Software 5.18 User Guide	MAN0026163
Ion S5™ Sequencer User Guide	MAN0017528
Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef User Guide	MAN0016854
Ion 540™ Kit – Chef User Guide	MAN0010851
Ion 550™ Kit – Chef User Guide	MAN0017275
Ion AmpliSeq™ & Ion AmpliSeq™ HD Custom Assay User Guide	MAN0028005
Ion AmpliSeq™ Designer Help	available at **AmpliSeq.com**

For additional documentation, see “Customer and technical support”.

Customer and technical support

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  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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 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**.