Ion AmpliSeq™ HD Library Kit

Catalog Numbers A57283, A37694, A37695, and A53690

Pub. No. MAN0017774 Rev. E.0

Note: For safety and biohazard guidelines, see the "Safety" appendix in the following product documentation: *Ion AmpliSeq™ HD Library Kit User Guide* (Pub. No. MAN0017392). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

This quick reference is intended as a benchtop reference for experienced users of the Ion AmpliSeq™ HD Library Kit with HD Enhancer (Cat. No. A57283). For detailed instructions, including for the legacy protocol without using 5X HD Enhancer, see the *Ion AmpliSeq™ HD Library Kit User Guide* (Pub. No. MAN0017392).

Prepare Ion AmpliSeq™ HD DNA libraries

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 "Remove deaminated bases from FFPE DNA" on page 2.

Note: If you are preparing 10X working FWD and REV subpools from 384-well plate format panels, see the *Ion AmpliSeq™ HD Library Kit User Guide*.

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 "Prepare Ion AmpliSeq™ HD RNA libraries" on page 7.

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.

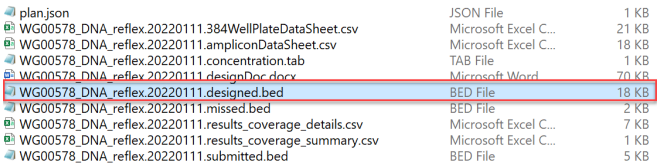

Figure 1 `designed.bed` **file**

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.

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 2.

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.
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 the *Ion AmpliSeq™ HD Library Kit User Guide*.
- Treat FFPE DNA samples with UDG before use. See "Remove deaminated bases from FFPE DNA" on page 2.

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 1 or the *Ion AmpliSeq™ HD Library Kit User Guide* (Plate format).

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 1)
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.

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

Proceed immediately to “Amplify the targets—DNA panel” on page 4.

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 the *Ion AmpliSeq™ HD Library Kit User Guide*.
- Treat FFPE DNA samples with UDG before use. See “Remove deaminated bases from FFPE DNA” on page 2.

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 1 or the *Ion AmpliSeq™ HD Library Kit User Guide* (Plate format).

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.

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

Proceed immediately to "Amplify the targets—DNA panel" on page 4.

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 4 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.

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.

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.

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 5.

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.

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 1	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 1 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:

- 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.
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.
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.

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 .

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.

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 7.

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.
- For legacy protocols without using 5X HD Enhancer, see the *Ion AmpliSeq™ HD Library Kit User Guide*.
- 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.

Proceed immediately to "Amplify the targets—RNA panel" on page 8

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 4 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.

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 9.

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.

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:

- 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.

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.

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.

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.

Note: For alternate quantification methods, see the *Ion AmpliSeq™ HD Library Kit User Guide*.

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.

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.

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).

1. Determine the molar concentration of the library using the Bioanalyzer™ software. 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.
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.
3. Dilute the library to 100 pM, then proceed to "Combine libraries" on page 11 or store libraries as described in "Store libraries" on page 11.

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.

Combine libraries

For instruction on how to combine libraries, and guidelines for templating, sequencing, and data analysis, see the *Ion AmpliSeq™ HD Library Kit User Guide* (Pub. No. MAN0017392).

Limited product warranty

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

Products manufactured at this site:
- Ion AmpliSeq™ HD Made-to-Order Panels

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

Products manufactured at this site:
- Ion AmpliSeq™ HD Library Kit
- Ion AmpliSeq™ HD Dual Barcode Kit 1–24

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

Revision history: Pub. No. MAN0017774 E.0

Revision	Date	Description
E.0	2 January 2024	• Updated to correspond to the *Ion AmpliSeq™ HD Library Kit User Guide* (Pub. No. MAN0017392) Rev F.0 • Added support for MagMAX™ Pure Bind Beads for purification. • Corrected information about preparing 10X DNA FWD and REV working subpools. Specified primer sizes, concentrations, and dilutions to use in both Tube and 384-well plate formats.
D.0	29 March 2023	• Support added for 5X HD Enhancer. • Updated protocol for Ion AmpliSeq™ HD DNA and RNA libraries.
C.0	8 December 2020	• Updated the DNA target amplification reaction setup to include the CRC reagent as a standard component in "Prepare molecular tagging reactions—1-pool primer panel" on page 2, and "Prepare molecular tagging reactions—2-pool primer panel" on page 3. • Removed the low concentration input reverse transcription reaction option in "Reverse transcribe the RNA" on page 7. • Added the required additional round of library purification steps in "Purify the DNA library" on page 6 and "Purify the RNA library" on page 9. • Updated method recommendation in "Quantify the library with the Agilent™ 2100 Bioanalyzer™ Instrument" on page 10.