Prepare Amplicon Libraries without Fragmentation Using the Ion Plus Fragment Library Kit

Publication Number MAN0006846 **Revision** B.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Overview

This user bulletin describes how to prepare Ion libraries from amplicons using the Ion Plus Fragment Library Kit (Cat. Nos. 4471252 and A28950) when no fragmentation is required. To prepare Ion libraries from amplicons that require fragmentation, see the *Prepare Amplicon Libraries Requiring Fragmentation Using the Ion Xpress™ Plus Fragment Library Kit User Bulletin* (Pub. No. MAN0007044).

IMPORTANT! The protocol does not cover preparation of Ion AmpliSeq™ libraries, which is described in the *Ion AmpliSeq™ DNA and RNA Library Preparation User Guide* (Pub. No. MAN0006735).

For information about using the Ion Plus Fragment Library Kit to prepare fragment libraries from genomic DNA, see the *Ion Xpress™ Plus gDNA Fragment Library Preparation User Guide* (Pub. No. MAN0009847).

Using the protocol in this user bulletin, first generate by PCR amplicons that are shorter than the median insert size for the desired Ion library. See "Guidelines for PCR primer design" on page 6 on page 6 for median insert size for the desired median adapter-ligated library size. The maximum amplicon length possible without need for fragmentation depends on the specific Ion template preparation and sequencing kits used during downstream procedures.

Following amplicon purification, use the Ion Plus Fragment Library Kit to end-repair the amplicons, ligate them to Ion-compatible adapters, and nick-repair to complete the linkage between the adapters and inserts. For barcoded libraries, substitute adapters from an Ion Xpress™ Barcode Adapters kit. Amplification of libraries is optional, depending on the amount of input DNA and your experimental requirements. Finally, proceed to library qualification, quantification and pooling, if barcoded libraries were prepared.

Template kit compatibility

These library kits are compatible with all current Ion template preparation kits for the Ion PGM™, Ion Proton™, Ion S5™, and Ion S5™ XL Systems.

Ion kits used in this protocol

Ion Plus Fragment Library Kits (10 and 48 reactions)

Use the Ion Plus Fragment Library Kit (Cat. No. 4471252), or the Ion Plus Fragment Library Kit 48 rxns (Cat. No. A28950), depending on your throughput requirements, to prepare fragment libraries from genomic DNA (gDNA) for downstream template preparation and sequencing on the Ion PGM™, Ion Proton™, Ion S5™, or Ion S5™ XL Systems.

Ion Plus Fragment Library Kit

The Ion Plus Fragment Library Kit (Cat. No. 4471252) provides reagents for preparing up to 10 libraries at 1 µg input, or up to 20 libraries at 100 ng input.

Contents	Cap color	Amount	Storage
5X End Repair Buffer	Red	400 µL	–30°C to –10°C
End Repair Enzyme	Orange	20 µL	
10X Ligase Buffer	Yellow	200 µL	
DNA Ligase	Blue	40 µL	
Nick Repair Polymerase	Clear	160 µL	
dNTP Mix	Violet	40 µL	
Adapters	Green	100 µL	
Platinum™ PCR SuperMix High Fidelity	Black	2 × 1000 µL	
Library Amplification Primer Mix	White	100 µL	
Low TE	Clear	2 × 1.5 mL	15°C to 30°C *or* –30°C to –10°C

Ion Plus Fragment Library Kit 48 rxns

The Ion Plus Fragment Library Kit 48 rxns (Cat. No. A28950) provides reagents for preparing up to 48 libraries at 1 µg input, or up to 96 libraries at 100 ng input.

Contents	Cap color	Amount	Storage
5X End Repair Buffer 48 rxns	Red	1.92 mL	–30°C to –10°C
End Repair Enzyme 48 rxns	Orange	96 µL	
10X Ligase Buffer 48 rxns	Yellow	960 µL	
DNA Ligase 48 rxns	Blue	192 µL	
Nick Repair Polymerase 48 rxns	Clear	768 µL	
dNTP Mix 48 rxns	Violet	192 µL	
Adapters 48 rxns	Green	480 µL	
Platinum™ PCR SuperMix High Fidelity 48 rxns	—	9.6 mL	
Library Amplification Primer Mix 48 rxns	White	480 µL	
Low TE 48 rxns	—	14.4 mL	15°C to 30°C *or* –30°C to –10°C

Optional: Ion Xpress™ Barcode Adapters Kits

The following Ion Xpress™ Barcode Adapters Kits are available:

- Ion Xpress™ Barcode Adapters 1–16 (Cat. No. 4471250)
- Ion Xpress™ Barcode Adapters 17–32 (Cat. No. 4474009)
- Ion Xpress™ Barcode Adapters 33–48 (Cat. No. 4474518)
- Ion Xpress™ Barcode Adapters 49–64 (Cat. No. 4474519)
- Ion Xpress™ Barcode Adapters 65–80 (Cat. No. 4474520)
- Ion Xpress™ Barcode Adapters 81–96 (Cat. No. 4474521)

Complete set of adapters:

- Ion Xpress™ Barcode Adapters 1–96 (Cat. No. 4474517)

Optional: Ion Plus Fragment Library Adaptors

The Ion Plus Fragment Library Adapters Kit (Cat. No. 4476340) contains additional adapters and Library Amplification Primer Mix, to prepare ≤20 libraries at 100 ng input, or ≤10 libraries at 1 µg input.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**www.fisherscientific.com**) or other major laboratory supplier.

Item	Source
Agencourt™ AMPure™ XP Kit[1]	Beckman Coulter A63880 or A63881
DynaMag™-2 magnet (magnetic rack) or Agencourt™ SPRIPlate 96R Super Magnet Plate	12321D NC9028329
Agilent™ 2100 Bioanalyzer™ instrument[2]	Agilent G2939AA
Agilent™ High Sensitivity DNA Kit[3]	Agilent 5067-4626
Optional: Ion Library TaqMan™ Quantitation Kit[4]	4468802
1.5-mL Eppendorf LoBind™ Tubes	Fisher Scientific 13-698-791
0.2-mL PCR tubes	MLS
Microcentrifuge	MLS
Thermal cycler	MLS
Vortex mixer	MLS
Pipettors 1–1000 µL	MLS
Barrier pipette tips	MLS
Nuclease-free Water	AM9932
Optional: 10 mM Tris, pH 7.5–8.5	MLS

[1] Required for DNA purification.

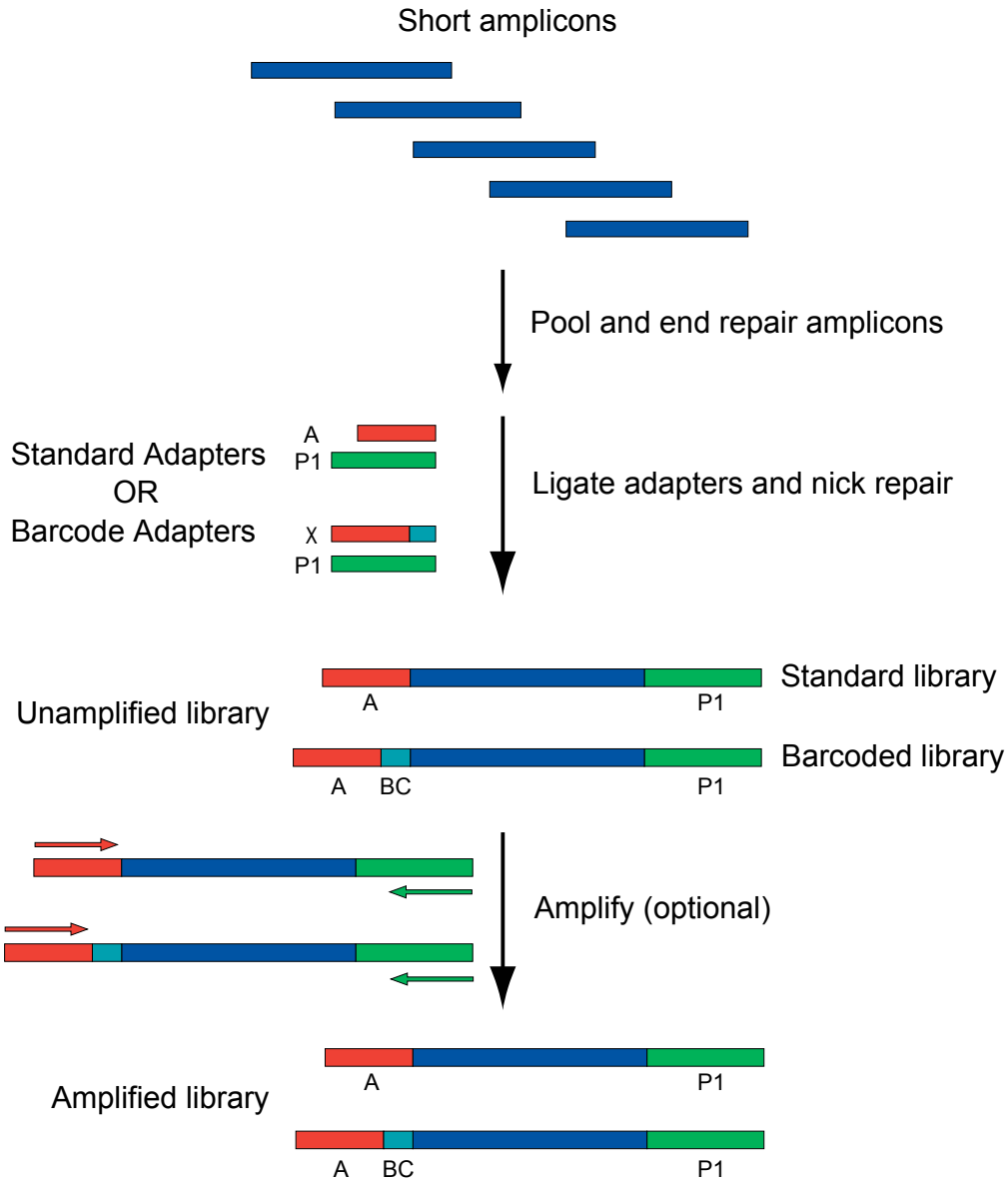
[2] Required for amplicon quantification and analysis of DNA fragment length distribution during library preparation, and *optional* library quantification.

[3] Required for amplicon quantification, and quality assessment and quantification of libraries with the Agilent™ 2100 Bioanalyzer™ instrument.

[4] Used for quantification of unamplified libraries in place of Agilent™ 2100 Bioanalyzer™ analysis.

Workflow

The procedure is identical for standard and barcoded libraries, except for the adapters used at the ligation and nick repair step. The average insert length of barcoded libraries is slightly shorter than of non-barcoded libraries to accommodate an additional 13 bp in the barcode adapter.



Procedural guidelines

Guidelines – general

- High-quality RNA-free DNA is required. The quality of the input DNA has a significant impact on the quality of the resulting library. A number of commercially available kits are available for isolation of high molecular weight, RNA-free genomic DNA. See "Evaluate the quality of the genomic DNA", Appendix C of the *Ion Xpress™ Plus gDNA Fragment Library Preparation User Guide* (Pub. No. MAN0009847), for more information about assessing the integrity and size of your input DNA material and performing an optional RNase treatment procedure.
- Use good laboratory practices to minimize cross-contamination of products. If possible, perform library construction in an area or room that is distinct from that of template preparation.
- When handling barcoded adapters, be especially careful not to cross-contaminate. Change gloves frequently and open one tube at a time.
- Perform all steps requiring 1.5-mL tubes with 1.5-mL Eppendorf LoBind™ Tubes.
- Thaw reagents on ice before use, and keep enzymes at –30°C to –10°C until ready to use.
- Mix reagents thoroughly before use, especially if frozen and thawed.

Guidelines for PCR primer design

- Use standard guidelines to design PCR primers for your region of interest. For design assistance, use a web tool such as Primer3, available at **http://primer3.ut.ee/**
- Design your primers so that any sequence variants of interest are located between the primers, so that those variants are not masked by the template-specific part of the primer sequences.
- When designing primers for amplicons not requiring fragmentation, design the primers so that the amplicons do not exceed the maximum insert size for the desired target read length of the library. If you would like to sequence through the entire insert, design the primers so that the amplicon does not exceed the target read length.

Library sizes for Ion PGM™ System and Ion S5™/Ion S5™ XL System sequencing

Target Read Length	Median Insert Size
400 bases (400-base-read library)	~410 bp
300 bases (300-base-read library)	~320 bp
200 bases (200-base-read library)	~260 bp
100 bases (100-base-read library)	~130 bp

Library sizes for Ion Proton™ System sequencing

Target Read Length	Median Insert Size
200 bases (200-base-read library)	~200 bp
150 bases (150-base-read library)	~150 bp

Guidelines for PCR

- Start with 20–50 ng of high-quality, RNA-free genomic DNA.
- Avoid overamplification, which can generate single-stranded DNA that cannot be fragmented properly for library construction.
- If 12 or more individually amplified amplicons will be pooled together for downstream library construction, consider using fewer amplification cycles (for example, 25–35 cycles rather than 40 cycles).
- We strongly recommend using a high-fidelity DNA polymerase to minimize amplification errors.

Guidelines for barcoding amplicon libraries

- A barcoded library typically represents one biological sample. The number of barcoded libraries that can be accommodated in a single sequencing run depends on the chip size, the size of the target region(s) of interest, and the coverage required.
- For a given chip and coverage depth, as the size of the target region to be sequenced decreases, the number of barcoded libraries that can be accommodated per sequencing run increases.

Prepare and purify amplicons

Example PCR and amplicon purification protocol

The following example procedure describes how to use Platinum™ PCR SuperMix High Fidelity to generate amplicons in a singleplex PCR, followed by purification using Agencourt™ AMPure™ XP Reagent. You may use this procedure or your standard laboratory procedure.

Materials required

- Forward and reverse PCR primers
- 0.2-mL PCR strip tubes or 96-well PCR plate
- Platinum™ PCR SuperMix High Fidelity
- Nuclease-free Water
- Agencourt™ AMPure™ XP Reagent
- SPRIPlate 96R Ring Magnet Plate
  or DynaMag™-2 magnet
- 70% ethanol, freshly prepared

Example PCR protocol

1. Thaw the PCR primers, Platinum™ PCR SuperMix High Fidelity, and high-quality genomic DNA on ice.

2. For each amplicon, mix equal volumes of the appropriate 10 µM forward and 10 µM reverse primers for a 10 µM primer stock mix (5 µM each primer).

3. Add the following reagents to 0.2-mL strip tubes or to the wells in a 96-well Eppendorf™ plate exactly in this order:

Component	Volume
Platinum™ PCR SuperMix High Fidelity[1]	45 µL
20–50 ng genomic DNA	4 µL
10 µM primer mix[2]	1 µL
Total	**50 µL**

[1] A 5-µL total volume of primer and template in a 50-µL reaction is optimum. No decrease in product yield is observed if the total volume of primer and template varies between 1 µL and 15 µL with 45 µL of Platinum™ PCR SuperMix High Fidelity.

[2] If larger volumes of primer mix are desired for pipetting, use 5 µL of a 2-µM primer mix. Adjust the volume of Platinum™ PCR SuperMix High Fidelity accordingly to keep the reaction volume at 50 µL.

4. Load the tubes or plate into a thermal cycler and run the program to amplify the genomic DNA targets.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	95°C	3 min
Cycle (40 cycles)	Denature	95°C	30 sec
	Anneal	58°C	30 sec
	Extend	68°C	1 min/kb
Hold	—	4°C	Up to 12 hours

Example amplicon purification protocol

Following amplification, purify the amplicons. We recommend using AMPure™ XP Reagent, as described in the following example protocol.

Elute or resuspend the purified amplicons in Nuclease-free Water.

IMPORTANT! If the total amplicon size, including target and primer sequence, is <100 bp, use a different purification method, such as the PureLink™ PCR Micro Kit (Thermo Fisher Scientific Cat. No. K310010), or the QIAGEN MinElute™ PCR Purification Kit.

1. Resuspend the Agencourt™ AMPure™ XP Reagent, and allow the suspension to come to room temperature (~30 minutes).

2. Prepare 70% ethanol: 70 µL per amplicon (includes 10 µL of overage per amplicon).

   IMPORTANT! Use freshly prepared 70% ethanol for the next steps. A higher percentage of ethanol causes inefficient washing of smaller-sized molecules. A lower percentage of ethanol could cause sample loss.

3. In each well or tube, add 90 µL (1.8X sample volume) Agencourt™ AMPure™ XP Reagent to the sample, pipet up and down to thoroughly mix the bead suspension with the DNA and incubate the mixture at room temperature for 5 minutes.

4. Place each plate or tube on a magnet (such as the DynaMag™-2 magnet or the Agencourt™ SPRIPlate 96R Super Magnet Plate) for 3 minutes or until the solution clears. Remove and discard the supernatant from each well or tube without disturbing the bead pellet.

5. Without removing the samples from the magnet, dispense 30 µL of freshly prepared 70% ethanol into each well or tube. Incubate the samples at room temperature for 30 seconds. After the solution clears, remove and discard the supernatant without disturbing the pellet.

6. Repeat step 5 for a second wash.

7. To remove residual ethanol, keep the sample on the magnet and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.

8. Keeping the sample on the magnet, air-dry the beads at room temperature for 3–5 minutes.

   IMPORTANT! Ensure that the pellet does not dry out completely.

9. Remove the plate or tubes from the magnet, and add 15 µL of Nuclease-free Water directly to each bead pellet to disperse the beads. Pipet the mixture up and down five times to mix thoroughly.

10. Place the plate or tubes on a magnet for at least 1 minute. After the solution clears, transfer the supernatant containing the purified amplicons to a new plate or tube without disturbing the pellet.

    IMPORTANT! The supernatant contains the amplicon DNA. Do not discard.

STOPPING POINT *(Optional)* Store the DNA at –30°C to –10°C.

Pool, end-repair, and purify the amplicons

This section describes preparing amplicons for ligation to Ion adapters.

Materials required

Required for pooling

- Agilent™ 2100 Bioanalyzer™ instrument
- Agilent™ High Sensitivity DNA Kit

Required for end repair and purification (provided in the Ion Plus Fragment Library Kit)

- 5X End Repair Buffer
- End Repair Enzyme

Other materials

- Nuclease-free Water
- 1.5-mL Eppendorf LoBind™ Tubes
- Agencourt™ AMPure™ XP Kit
- DynaMag™–96 Side Magnetic rack

Prepare an equimolar pool of amplicons

Pooling amplicons in equimolar amounts for Ion library construction ensures even coverage of the target regions.

1. Using your laboratory practices or those described in the previous example protocol, amplify gDNA targets of interest from 20–50 ng gDNA and purify the individual amplicons. Use Nuclease-free Water for the final amplicon elution or resuspension.

2. Prepare an equimolar pool of purified amplicons at the highest possible concentration.
   a. Analyze 1 µL of each amplicon using an Agilent™ 2100 Bioanalyzer™ instrument and Agilent™ High Sensitivity DNA Kit. Follow the manufacturer's instructions.

   b. Use the Bioanalyzer™ software to determine the molar concentration (nmol/L) of each amplicon.

   c. Combine equimolar amounts of each amplicon stock. If you dilute the stocks before pooling, use Nuclease-free Water or 10 mM Tris, pH 7.5–8.5 to prepare the diluted amplicon stocks.

3. Calculate the combined concentration of the pooled amplicons, and convert the concentration of the pooled amplicon stock to ng/µL.

   Alternatively, analyze 1 µL of the pooled DNA with the Agilent™ 2100 Bioanalyzer™ instrument and an Agilent™ High Sensitivity DNA Kit, and use the Bioanalyzer™ software to determine the molar concentration of the amplicon pool. If necessary, use manual integration to place the entire range of amplicons within a single peak. Follow the manufacturer's instructions.

STOPPING POINT (Optional) Store the pooled amplicon stock at –30°C to –10°C. Before use, thaw the amplicon stock on ice. To reduce the number of freeze-thaw cycles, store the amplicon stocks in several aliquots.

End repair and purify the pooled amplicons

End repair

Note: Before use, pulse-spin components of the Ion Plus Fragment Library Kit for 2 seconds to collect the contents in the bottom of the tubes.

1. Prepare 10–100 ng of the amplicon pool in a total volume 79 µL of Nuclease-free Water.

2. Mix by pipetting in a 1.5-mL Eppendorf LoBind™ Tube:

Component	Volume
Pooled amplicons, 10–100 ng	79 µL
5X End Repair Buffer (red cap)	20 µL
End Repair Enzyme (orange cap)	1 µL
Total	**100 µL**

3. Incubate the end repair reaction for 20 minutes at room temperature.

Purify the pooled amplicons

Purify the end-repaired DNA with the Agencourt™ AMPure™ XP Kit:

IMPORTANT! Use freshly prepared 70% ethanol for the next steps. A higher percentage of ethanol causes inefficient washing of smaller-sized molecules. A lower percentage of ethanol could cause sample loss.

1. Add 180 µL of Agencourt™ AMPure™ XP Reagent (1.8X sample volume) to the sample, pipet up and down five times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate at room temperature 5 minutes.
2. Pulse-spin and place the sample tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution clears. Remove and discard the supernatant without disturbing the bead pellet.
3. Without removing the tube from the magnet, dispense 500 µL of freshly prepared 70% ethanol to the sample.
4. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
5. Repeat steps 3 and 4 for a second wash.
6. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
7. Keeping the tube on the magnet, air-dry the beads at room temperature for 3–5 minutes.
8. Remove the tube from the magnet, and add 25 µL of Low TE to the sample. Pipet the mixture up and down five times, then vortex the sample for 10 seconds to mix thoroughly.
9. Pulse-spin and place the tube in the magnetic rack for at least 1 minute. After the solution clears, transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind™ Tube without disturbing the pellet.

   IMPORTANT! The supernatant contains the eluted DNA. **Do not discard.**

   STOPPING POINT *(Optional)* Store the DNA at –30°C to –10°C.

10. Proceed to "Ligate adaptors, nick repair, and purify" on page 13.

Ligate adaptors, nick repair, and purify

Materials required

Materials provided in the Ion Plus Fragment Library Kit

- 10X Ligase Buffer
- Adapters *or* Ion P1 Adapter and Ion Xpress™ Barcode X adapter
- dNTP Mix
- DNA Ligase
- Nick Repair Polymerase

Other materials

- Thermal cycler
- 0.2-mL PCR tubes
- 1.5-mL Eppendorf LoBind™ Tube
- Agencourt™ AMPure™ XP Kit
- Freshly prepared 70% ethanol
- Magnetic rack

Ligate and nick repair

1. In a 0.2-mL PCR tube, combine the reagents as indicated in the appropriate table for non-barcoded or barcoded libraries, and mix well by pipetting up and down.

Non-barcoded libraries		Barcoded Libraries	
Component	**Volume**	**Component**	**Volume**
DNA	~25 µL	DNA	~25 µL
10X Ligase Buffer	10 µL	10X Ligase Buffer	10 µL
Adapters	2 µL	Ion P1 Adapter	2 µL
		Ion Xpress™ Barcode X[1]	2 µL
dNTP Mix	2 µL	dNTP Mix	2 µL
Nuclease-free Water	51 µL	Nuclease-free Water	49 µL
DNA Ligase	2 µL	DNA Ligase	2 µL
Nick Repair Polymerase	8 µL	Nick Repair Polymerase	8 µL
Total	**100 µL**	**Total**	**100 µL**

[1] X = barcode chosen.

Note: For barcoded libraries, add both Ion P1 Adapter and the desired Ion Xpress™ Barcode X adapter to the ligation reaction.

IMPORTANT! When handling barcoded adapters, be especially careful not to cross-contaminate. Change gloves frequently and open one tube at a time.

2. Place the tube in a thermal cycler and run the following program.

Stage	Temperature	Time
Hold	25°C	15 min
Hold	72°C	5 min
Hold	4°C	up to 1 h[1]

[1] Remove sample when ready to proceed to the next step. The last stage is not a stopping point; continue directly to the purification step.

3. Transfer the entire reaction mixture to a 1.5-mL Eppendorf LoBind™ Tube for the next cleanup step.

Purify the adapter-ligated and nick-repaired DNA

IMPORTANT! Use freshly prepared 70% ethanol (1 mL plus overage per sample) for the next steps.

1. Add the indicated volume of Agencourt™ AMPure™ XP Reagent to the sample, pipet up and down five times to thoroughly mix the bead suspension with the DNA, pulse-spin the tube, and incubate the mixture for 5 minutes at room temperature.

Library Size	Volume of AMPure™ XP Reagent
400-base-read	100 µL (1X sample volume)
200–300-base-read	120 µL (1.2X sample volume)
100–150-base-read	150 µL (1.5X sample volume)

2. Pulse-spin and place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.

3. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol.

4. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.

5. Repeat steps 3 and 4 for a second wash.

6. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.

7. Keeping the tube on the magnetic rack, air-dry the beads at room temperature for 3–5 minutes.

8. Remove the tube from the magnetic rack and add 20 µL of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down five times, then vortex the sample for 10 seconds to mix thoroughly.

9. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind™ Tube without disturbing the pellet.

   IMPORTANT! The supernatant contains the eluted DNA. **Do not discard.**

   STOPPING POINT *(Optional)* Store the DNA at –30°C to –10°C.

10. Proceed to "Determine if library amplification is required".

Determine if library amplification is required

Estimate the number of template preparation reactions that can be performed with the unamplified library, to determine if the yield of the unamplified library is sufficient for your experimental needs.

Note: Amplification is recommended for Ion Proton™ System sequencing.

1. Quantify the unamplified library by qPCR with the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802). This kit directly determines the library concentration so that a dilution to 100 pM may be made for template preparation. Follow the instructions in the *Ion Library TaqMan™ Quantitation Kit User Guide* (Pub. No. MAN0015802), and prepare a 1:1000 dilution of the unamplified library for qPCR.

2. Calculate the number of template preparation reactions that can be performed with the unamplified library as follows:

   Number of reactions = [(library volume in µL) × (library concentration in pM ÷ 100 pM)] ÷ [volume per template preparation reaction in µL]

   For the volume per template preparation reaction, see the specific user guide for the appropriate template preparation kit.

   If the estimated number of template preparation reactions is sufficient for your experimental requirements, no amplification is necessary.

3. Proceed to either amplify or further qualify the library, according to your experimental needs.

Library Amplification	Proceed to...
Yes	"Amplify and purify the library" on page 16
No	"Qualify non-barcoded libraries" on page 18 *or* "Qualify and pool barcoded libraries" on page 20

Amplify and purify the library

Materials required

Materials provided in the Ion Plus Fragment Library Kit

- Platinum™ PCR SuperMix High Fidelity
- Library Amplification Primer Mix
- Low TE

Other materials

- Thermal cycler
- 0.2-mL PCR tubes
- 1.5-mL Eppendorf LoBind™ Tube
- Agencourt™ AMPure™ XP Kit
- Freshly prepared 70% ethanol
- Magnetic rack

Amplify the library

1. Add 5 µL of Low TE to the ~20 µL of purified, adapter-ligated library.
2. Combine the following reagents in an appropriately sized tube and mix by pipetting up and down.

Component	Volume
Platinum™ PCR SuperMix High Fidelity	100 µL
Library Amplification Primer Mix	5 µL
Unamplified library	25 µL
Total	**130 µL**

3. Split the 130-µL reaction into two 0.2-mL PCR tubes, each containing about 65 µL.
4. Place the tubes into a thermal cycler and run the following PCR cycling program.

   Note: Minimize the number of cycles to avoid over-amplification, production of concatemers, and introduction of PCR-induced errors. Reduce the number of cycles if concatemers are formed.

Stage	Step	Temperature	Time
Hold	Denature	95°C	5 min
Cycle (5–7 cycles[1])	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	70°C	1 min
Hold	—	4°C	Hold for up to 1 hour

[1] 5 cycles for 50 ng of input, 7 cycles for 20 ng of input.

5. Combine previously split PCRs in a new 1.5-mL Eppendorf LoBind™ Tube.

IMPORTANT! Use **freshly prepared 70% ethanol** (1 mL plus overage per sample) for the next steps.

Purify the library

1. Add 195 µL of Agencourt™ AMPure™ XP Reagent (1.5X sample volume) to each sample, pipet up and down five times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate the mixture for 5 minutes at room temperature.
2. Pulse-spin and place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.
3. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol.
4. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
5. Repeat steps 3 and 4 for a second wash.
6. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
7. Keeping the tube on the magnet, air-dry the beads at room temperature for 3–5 minutes.
8. Remove the tube from the magnetic rack, and add 20 µL of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down five times, then vortex the sample for 10 seconds, to mix thoroughly.
9. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind™ Tube without disturbing the pellet.

   IMPORTANT! The supernatant contains the final amplified library. **Do not discard.**

10. To remove residual beads from the eluted DNA, place the tube with the eluted DNA back on the magnet for at least 1 minute, and transfer the supernatant to a new 1.5-mL Eppendorf LoBind™ Tube without disturbing the pellet.

    STOPPING POINT Store the library at –30°C to –10°C. Before use, thaw on ice. To reduce the number of freeze-thaw cycles, store the library in several aliquots.

11. **For non-barcoded libraries**, proceed to "Qualify non-barcoded libraries" on page 18.
    For barcoded libraries, proceed to "Qualify and pool barcoded libraries" on page 20.

Qualify non-barcoded libraries

Assess the quality of the library

Analyze an aliquot of the amplified or unamplified library on the 2100 Bioanalyzer™ instrument with an Agilent™ High Sensitivity DNA Kit, according to the following table.

Unamplified aliquot	Amplified aliquot
1 µL, undiluted	1 µL, diluted 1:10

IMPORTANT! Ensure that excessive amounts of primer-dimers (immediately adjacent to the marker) or overamplification products (concatemers) are not present. For more information, contact Technical Support.

Determine the library dilution required for template preparation

Quantify the library and determine the library dilution that results in a concentration within the optimal range for template preparation.

- **Unamplified libraries:** Determine the library dilution by qPCR with the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802).
- **Amplified libraries:** Determine the library dilution by Agilent™ 2100 Bioanalyzer™ instrument analysis or by qPCR.

Quantitation Method	Features
Ion Library TaqMan™ Quantitation Kit (qPCR)	• Quantitative real-time PCR (qPCR) methodology. • Direct determination of the library concentration from a standard curve. • Higher precision for quantitation. A single dilution of the library is usually sufficient for an optimized template preparation procedure. • Higher sensitivity for detection. The Ion Library TaqMan™ Quantitation Kit is recommended for unamplified or low-yield libraries. Libraries with insufficient material for detection by the Agilent™ 2100 Bioanalyzer™ instrument may have material that is detectable by qPCR and sufficient for sequencing. • Unamplified and low-yield libraries also contain unadapted and improperly adapted fragments. The Ion Library TaqMan™ Quantitation Kit accurately quantifies the properly adapted libraries with minimal impact from background material
Agilent™ 2100 Bioanalyzer™ instrument analysis	• Used to determine a molar concentration of the library, from which the library dilution is calculated. • Concentration is part of the output of Bioanalyzer™ instrument analysis to assess the quality, so an additional quantitation procedure is unnecessary. • Lower precision for quantitation. Titration of the library over a 4-fold concentration range based on Bioanalyzer™ instrument analysis must be performed for optimized template preparation.

If you perform both procedures:

- Use the Ion Library TaqMan™ Quantitation Kit to determine the library dilution.
- Use Agilent™ 2100 Bioanalyzer™ instrument analysis to assess the quality of the library.

Determine library concentration using the Ion Library TaqMan™ Quantitation Kit (for amplified or unamplified libraries)

1. Use the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802) to determine the library concentration in pmol/L by quantitative real-time PCR (qPCR). Follow the instructions in the *Ion Library TaqMan™ Quantitation Kit User Guide* (Pub. No. MAN0015802).

2. Dilute the library to a concentration of ~100 pM. This concentration is suitable for downstream template preparation.

   Determine the dilution factor using the following formula:

   Dilution factor = (Library concentration in pM)/100 pM

   Example:

   The library concentration is 15,000 pM.

   Dilution factor = 15,000 pM/100 pM = 150

   Thus, 1 µL of library diluted with 149 µL of Low TE (1:150 dilution) yields a 100 pM solution. Use this library dilution for template preparation.

   Note: If you previously quantified an unamplified library with the Ion Library TaqMan™ Quantitation Kit and did not amplify the library, you do not need to repeat the qPCR.

Determine the library concentration from Agilent™ Bioanalyzer™ 2100 instrument analysis (amplified libraries only)

1. From the Agilent™ Bioanalyzer™ 2100 instrument analysis used to assess the library size distribution, determine the molar library concentration in pmol/L using the Bioanalyzer™ 2100 software. If necessary, follow the manufacturer's instructions to perform a region analysis (smear analysis) to place the entire distribution of library molecules within a single peak.

2. Dilute the library to a concentration of ~100 pM. This concentration is suitable for downstream template preparation.

   Determine the dilution factor using the following formula:

   Dilution factor = (Library concentration in pM)/100 pM

   Example:

   The library concentration is 15,000 pM.

   Dilution factor = 15,000 pM/100 pM = 150

   Thus, 1 µL of library diluted with 149 µL of Low TE (1:150 dilution) yields a 100 pM solution. Use this library dilution for template preparation.

   Note: Because Bioanalyzer™ 2100 instrument quantification is not as precise as qPCR, when you perform the template preparation procedure, you will need to prepare 3 serial dilutions of the library at 0.5X library dilution (~50 pM), 1X library dilution (~100 pM), and 2X library dilution (~200 pM) to ensure that one or more dilutions are in the optimal concentration range.

Proceed to template preparation

Prior to template preparation, dilute an appropriate aliquot of each library (based on your template kit requirements) using the calculations above.

Note: Diluted libraries should be stored at 2°C to 8°C and used within 48 hours. Store undiluted libraries at –30°C to –10°C.

The libraries are ready for downstream template preparation using an appropriate Ion template preparation kit.

Qualify and pool barcoded libraries

Pooling barcoded libraries in equimolar amounts ensures equal representation of each barcoded library in the sequencing run. This section describes alternative pooling procedures according to the library quantification method.

Note: Unamplified libraries must be quantified for pooling with the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802).

For non-barcoded libraries, go to "Qualify non-barcoded libraries" on page 18.

Assess the quality of individual barcoded libraries

Analyze an aliquot of the amplified or unamplified library on the 2100 Bioanalyzer™ instrument with an Agilent™ High Sensitivity DNA Kit, according to the following table.

Unamplified aliquot	Amplified aliquot
1 µL, undiluted	1 µL, diluted 1:10

IMPORTANT! Ensure that excessive amounts of primer-dimers (immediately adjacent to the marker) or overamplification products (concatemers) are not present. For more information, contact Technical Support.

Individual barcoded libraries display the same size distributions as non-barcoded libraries.

Pool barcoded libraries

Pool barcoded libraries using qPCR (unamplified libraries or amplified libraries)

1. Use the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802) to determine library concentration in pmol/L by quantitative real-time PCR (qPCR) for each individual barcoded library. Follow the instructions in the *Ion Library TaqMan™ Quantitation Kit User Guide* (Pub. No. MAN0015802).

2. Dilute each barcoded library to a concentration of ~100 pM. This concentration is suitable for downstream template preparation.

   Determine the dilution factor using the following formula:

   Dilution factor = (Barcoded library concentration in pM)/100 pM

   Example:

   The barcoded library concentration is 15,000 pM.

   Dilution factor = 15,000 pM/100 pM = 150

   Thus, 1 µL of barcoded library diluted with 149 µL of Low TE (1:150 dilution) yields a 100 pM solution.

3. Prepare at least 20 µL of a barcoded library pool by mixing equal volumes of the diluted barcoded libraries. The library pool will be at the correct concentration for template preparation using Ion template preparation kits.

Pool barcoded libraries using Bioanalyzer™ instrument quantification (amplified libraries only)

1. Using Bioanalyzer™ software, determine the molar concentration in pmol/L of each barcoded library from the Agilent™ 2100 Bioanalyzer™ instrument analysis used to assess the individual barcoded library size distribution.

   Note: If necessary, follow the manufacturer's instructions to perform a region analysis (smear analysis) to place the entire distribution of library molecules within a single peak.

2. Prepare an equimolar pool of barcoded libraries at the highest possible concentration.

   STOPPING POINT *(Optional)* Store the library pool at –30°C to –10°C. To reduce the number of freeze-thaw cycles, store the library pool in several aliquots. Thaw on ice.

3. Determine the molar concentration of the library pool.
   - Use the combined concentration of the library pool calculated for your library pooling algorithm.
   - Alternatively, confirm the concentration of the library pool by analyzing 1 µL of the library pool on the Bioanalyzer™ instrument with an Agilent™ High Sensitivity DNA Kit.

     Determine the molar concentration of the library pool using the Bioanalyzer™ software. If necessary, follow the manufacturer's instructions to perform a region analysis (smear analysis) to place the entire distribution of library molecules within a single peak.

4. Dilute the library pool to a concentration of ~100 pM. This concentration is suitable for downstream template preparation.

   Determine the dilution factor using the following formula:

   Dilution factor = (Library pool concentration in pM)/100 pM

   Example:

   The library pool concentration is 15,000 pM.

   Dilution factor = 15,000 pM/100 pM = 150

   Thus, 1 µL of library pool diluted with 149 µL of Low TE (1:150 dilution) yields a 100 pM solution. Use this library dilution for template preparation.

Proceed to template preparation

Prior to template preparation, dilute an appropriate aliquot of each library (based on your template kit requirements) using the calculations above.

Note: Diluted libraries should be stored at 2°C to 8°C and used within 48 hours. Store undiluted libraries at –30°C to –10°C.

The libraries are ready for downstream template preparation using an appropriate Ion template preparation kit.

Supplemental information

Barcode discrimination

Torrent Suite™ Software v5.0 or later is recommended for sequence data analysis. The software includes tools for analysis of barcoded libraries prepared with the Ion Xpress™ Barcode Adapters 1-96.

The Ion Xpress™ Barcode Adapters 1-96 were designed for clear separation in flowspace. Barcodes are correctly assigned with high confidence in reads with ≤2 flowspace errors in the barcode region. In the rare situation of reads with ≥3 in the barcode region, barcodes could be misassigned. The number of allowable errors can be reduced from 2 to 1 or 0 in the Torrent Suite™ Software to reduce the risk of barcode misassignment; however, the number of reads assigned to a barcode will be reduced concomitantly.

In general practice, the chance of barcode misassignment is much less than that of adapter, library, or templated Ion Sphere™ Particle cross-contamination. For experiments in which even a low degree of cross-contamination (<1%) will be detrimental, users are advised to take measures to avoid exposure of library reagents to amplified products, particularly after the template preparation procedure.

Ion non-barcoded and barcode adapter sequences

In each sequence, a "*" indicates a phosphorothioate bond, for protection from nucleases and to preserve the directionality of adapter ligation.

Non-barcoded A adapter and P1 adapter sequences

```
Ion A Adapter (non-barcoded)
   1     CCATCT CATCCCTGCG TGTCTCCGAC TCAG
   1 T*T*GGTAGA GTAGGGACGC ACAGAGGCTG AGTC
```

```
Ion P1 Adapter
   1     CCACTA CGCCTCCGCT TTCCTCTCTA TGGGCAGTCG GTGAT
   1 T*T*GGTGAT GCGGAGGCGA AAGGAGAGAT ACCCGTCAGC CACTA
```

Barcode (A) adapter sequences

- Barcode (A) adapter sequences are available on the Ion Community. Visit the Ion Community at **ioncommunity.thermofisher.com** and perform a search for "Ion 96 Barcode Set."

Documentation and support

Customer and technical support

Visit **thermofisher.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
  - Product FAQs
  - Software, patches, and updates
- Order and web support
- Product documentation, including:
  - 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 found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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

DISCLAIMER

TO THE EXTENT ALLOWED BY LAW, LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Revision history: Pub. No. MAN0006846

Revision	Date	Description of Change
B.0	24 April 2016	• Added support for 48-reaction Ion Plus Fragment Library Kit • Added support for Ion S5™ and Ion S5™ XL Systems • General rebranding and streamlining
A.0	3 March 2014	• Added support for 400-base-read libraries • Added support for the Ion Proton™ System. • Version numbering changed to alphanumeric format and reset to A.0 in conformance with internal document control procedures

Corporate entity: Life Technologies Corporation | Carlsbad, CA 92008 USA | Toll Free in USA 1 800 955 6288

For support visit **thermofisher.com/support** or email **techsupport@lifetech.com**

thermofisher.com

ThermoFisher SCIENTIFIC

24 April 2016