Prepare Amplicon Libraries Requiring Fragmentation Using the Ion Xpress™ Plus Fragment Library Kit

Publication Number MAN0007044 **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**.

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Overview

This user bulletin describes how to prepare Ion libraries from amplicons requiring fragmentation using the Ion Xpress™ Plus Fragment Library Kit (Cat. No. 4471269). To prepare Ion libraries from amplicons that do not require fragmentation, see the *Prepare Amplicon Libraries without Fragmentation Using the Ion Plus Fragment Library Kit User Bulletin* (Pub. No. MAN0006846).

IMPORTANT! The protocol does not cover preparation of Ion AmpliSeq™ libraries, which is described in the *Ion AmpliSeq™ Library Kit 2.0 User Guide* (Pub. No. MAN0006735).

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

Using the protocol in this user bulletin, first generate by PCR amplicons that are at least five times longer than the targeted library insert length for efficient fragmentation. For a 200-base-read library, for example, amplicon size should be at least 1000 bp for efficient fragmentation and good coverage uniformity. Following amplicon purification, pool the amplicons and shear with Ion Shear™ Plus reagents and re-purify. Ligate amplicons to Ion-compatible adapters, and nick-repair to complete the linkage between the adapters and inserts. For barcoded libraries, substitute adapters from a Ion Xpress™ Barcode Adapters kit.

Purify, then size-select the adapter-ligated library for optimum length according to target read length, as shown in the following table:

Target Read Length	Median Insert Size	Median Library Size (adapter-ligated)
Library sizes for Ion PGM™ System and Ion S5™/Ion S5™ XL System sequencing		
400 bases (400-base-read library)	~410 bp	~480 bp
300 bases (300-base-read library)	~320 bp	~390 bp
200 bases (200-base-read library)	~260 bp	~330 bp
100 bases (100-base-read library)	~130 bp	~200 bp
Library sizes for Ion Proton™ System sequencing		
200 bases (200-base-read library)	~200 bp	~270 bp
150 bases (150-base-read library)	~150 bp	~220 bp

After size selection, amplify and purify the library. Amplification is optional, depending on the amount of input DNA and your experimental requirements. Quantify the library and dilute for template preparation.

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 Xpress™ Plus Fragment Library Kit

The Ion Xpress™ Plus Fragment Library Kit (Cat. No. 4471269) contains two boxes: the Ion Shear™ Plus Reagents Kit and the Ion Plus Fragment Library Kit (listed below).

Supplied reagents are sufficient for preparing ≤20 libraries at 100 ng input, and ≤10 libraries at 1 µg input. The kit contains the following components:

Contents	Cap color	Amount	Storage
Ion Shear™ Plus Reagents Kit			
Ion Shear™ Plus 10X Reaction Buffer	Clear	2 × 50 µL	–30°C to –10°C
Ion Shear™ Plus Enzyme Mix II [1]	Clear	2 × 100 µL	
Ion Shear™ Plus Stop Buffer	Clear	2 × 50 µL	
Ion Plus Fragment Library Kit			
5X End Repair Buffer	Red	400 µL	–30°C to –10°C
End Repair Enzyme[2]	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

[1] Ion Shear™ Plus Enzyme Mix II is an improved formulation of Ion Shear™ Plus Enzyme Mix.

[2] 5X End Repair Buffer and End Repair Enzyme are required only for physically fragmented gDNA.

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)	12321D
Agilent™ 2100 Bioanalyzer™ instrument[2]	Agilent G2939AA
Agilent™ High Sensitivity DNA Kit	Agilent 5067-4626
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: Ion Library TaqMan™ Quantitation Kit (required for quantification of unamplified libraries)	4468802

Item	Source
Optional: Ion Ion PGM™ Controls Kit, Ion PGM™ Controls Kit v2, Ion PI™ Controls 200 Kit, *or* Ion S5™ Controls Kit	4480449, 4482010, 4488985, or A27760
Optional: 10 mM Tris, pH 7.5–8.5	MLS

[1] Use the Agencourt™ AMPure™ XP Kit for DNA purification.

[2] Use the Agilent™ 2100 Bioanalyzer™ instrument to analyze DNA fragment length distribution during library preparation.

Required for size-selection using E-Gel™ SizeSelect™ Gels

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Item	Source
E-Gel™ iBase™ unit and E-Gel™ Safe Imager™ transilluminator combo kit	G6465
E-Gel™ SizeSelect™ 2% Agarose	G661002
50-bp DNA ladder (1 µg/µL), required for 100-, 150-, 200- and 400-base-read libraries	10416014
100-bp DNA ladder (1 µg/µL), required for 300-base-read libraries	15628019

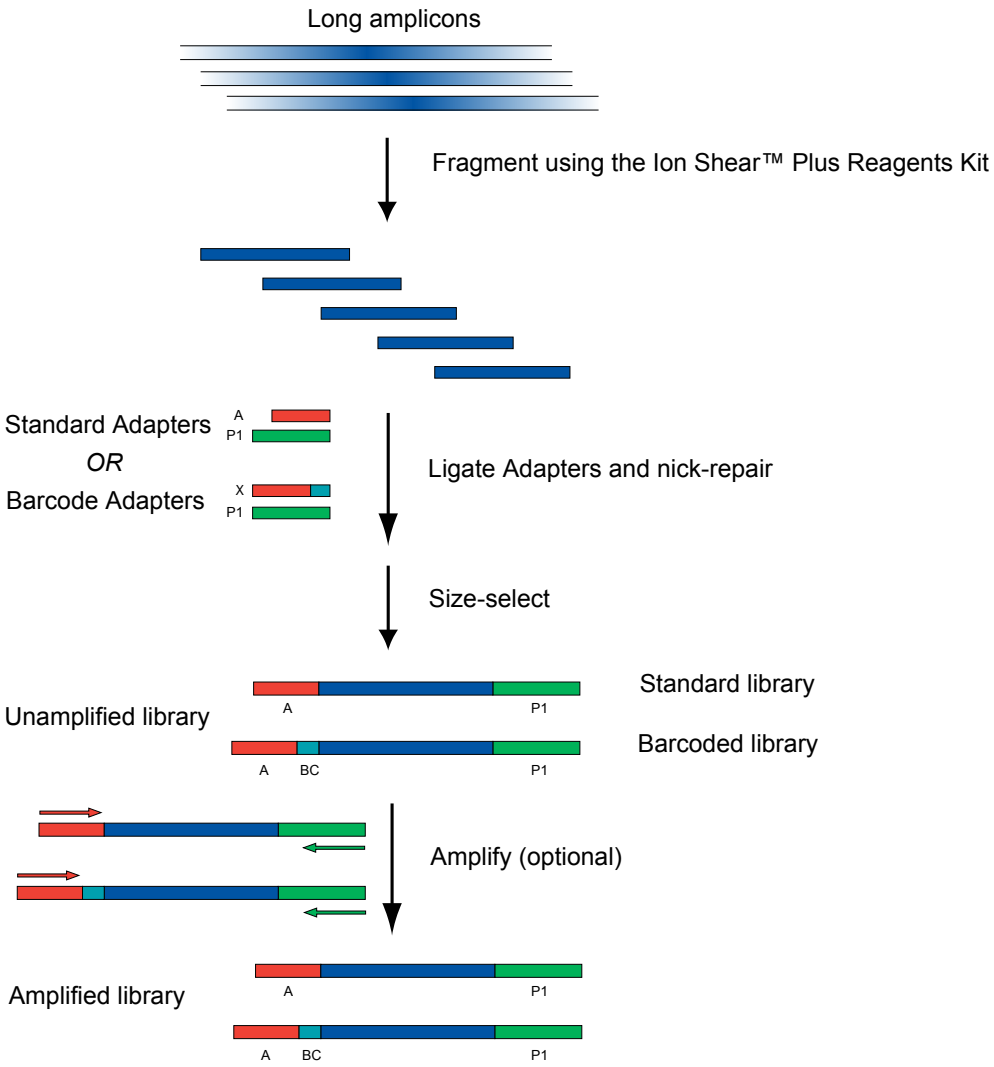
Required for size-selection using the Pippin Prep™ instrument

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Item	Source
Pippin Prep™ instrument	4471271
2% Agarose Gel Cassettes for the Pippin Prep™ instrument	4472170

Workflow overview

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

- 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 reduce amplification errors.
- For amplicons 1–8 kbp in length, determine PCR conditions empirically or use the SequalPrep™ Long PCR Kit with dNTPs (Cat. No. A10498).

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, keep in mind that the primer sequence will not yield valuable sequence information, and the coverage of the ends of amplicons—i.e., the first 50 bases—is on average one-half of the average coverage.
- We recommend that amplicon length be at least five times longer than the targeted library insert length for efficient fragmentation. For a 200-base-read library, for example, amplicon size should be at least 1000 bp for efficient fragmentation and good coverage uniformity.

Guidelines for purification

Following amplification, purify the amplicons.

- We recommend using Agencourt™ AMPure™ XP Reagent, as described in the following example protocol.
- Elute or resuspend the purified amplicons in Nuclease-free Water.

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.

1. Resuspend the Agencourt™ AMPure™ XP Reagent, and allow the mixture 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 Agencourt™ SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator) 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.

   IMPORTANT! For amplicons that will be fragmented using the Ion Shear™ Plus Reagents, it is important to elute the amplicon DNA in Nuclease-free Water. EDTA can significantly interfere with the Ion Shear™ reaction.

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, fragment, and purify the amplicons

This section describes how to pool, fragment, and purify the amplicons prior to ligation to Ion adapters.

Materials required

Required for pooling

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

Required for fragmentation and purification (provided in the Ion Xpress™ Plus Fragment Library Kit)

- Ion Shear™ Plus 10X Reaction Buffer
- Ion Shear™ Plus Enzyme Mix II
- Ion Shear™ Plus Stop Buffer
- Low TE

Other materials and equipment

- Nuclease-free Water
- 1.5-mL Eppendorf LoBind™ tubes
- 0.2-ml PCR tubes
- 37°C heat block/water bath
- P10–P20 and P100–P200 pipettors
- Ice
- Agencourt™ AMPure™ XP Kit
- Freshly prepared 70% ethanol
- Magnetic rack
- *(Optional) E. coli* DH10B Control DNA or Human CEPH DNA Control

Prepare an equimolar pool of amplicons

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

IMPORTANT! EDTA-containing buffers can significantly interfere with the Ion Shear™ Plus reaction. Use Nuclease-free Water or 10 mM Tris, pH 7.5–8.5, for the final amplicon elution or resuspension and to prepare the amplicon pool.

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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 supplier'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 supplier'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.

Guidelines using Ion Shear™ Plus reagents

- Choose the fragmentation conditions according to the desired library size. The fragmented DNA is ready for adapter ligation.

  Library sizes for Ion PGM™ System or 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

- The Ion Shear™ Plus reaction has very good tolerance on the G+C content of a sample. However, the Ion Shear™ reaction is very sensitive to EDTA concentration, the integrity of the sample, and operator handling method.
- The final EDTA concentration must be ≤0.1 mM in the DNA preparation for the Ion Shear™ Plus reaction in step 3. If necessary, ethanol-precipitate the appropriate amount of the DNA preparation and resuspend in Nuclease-free Water or 10 mM Tris, pH 7.5–8 for this procedure.
- *(Optional)* Prepare a control sample in a separate tube. Use 1 µL (100 ng) of E. coli DH10B Genomic DNA Control for the Ion PGM™ System, or 1 µL (100 ng) of Human CEPH Genomic DNA Control for the Ion Proton™ System or the Ion S5™ XL System/Ion S5™ XL System, mixed with 9 µL of Nuclease-free Water or 10 mM Tris, pH 7.5–8.5.

Fragment the DNA

1. From the mass concentration calculated or determined during the pooling procedure, determine the volume for 100 ng.

2. Vortex the Ion Shear™ Plus 10X Reaction Buffer and the Ion Shear™ Plus Enzyme Mix II each for 5 seconds, pulse-spin to bring the contents to the bottom of the tubes, and place on ice.

   IMPORTANT! Thoroughly mix the Ion Shear™ Plus 10X Reaction Buffer and the Ion Shear™ Plus Enzyme Mix II individually before dispensing them in the next steps.

3. Add the following reagents in the indicated order to a 1.5-mL Eppendorf LoBind™ Tube, and mix vigorously by vortexing for 5 seconds. Pulse-spin to bring the contents to the bottom of the tube.

   Note: Do not scale up the reaction volumes or prepare a master mix.

Component	Volume
Pooled amplicons, 100 ng	*Y* µL
Ion Shear™ Plus 10X Reaction Buffer	5 µL
Nuclease-free Water	35 – *Y* µL
Total	**40 µL**

4. Using a P10–P20 pipettor, add 10 µL Ion Shear™ Plus Enzyme Mix II to the sample, for a total volume of 50 µL. Proceed immediately to the next step to mix the enzyme mix with the DNA and buffer.

5. Using a P100–P200 pipettor set at a 40-µL volume, mix the reaction by rapidly pipetting up and down eight to ten times. **Do not mix by vortexing and avoid creating bubbles.**

6. Incubate the tube(s) in a water bath or heat block at 37°C for the indicated reaction time.

   IMPORTANT! The Ion Shear™ reaction is very sensitive to sample integrity and operator handling method. The reaction time can be optimized under your laboratory conditions within the reaction times indicated in the following table.

Median Fragment Size	Reaction Time	Optimization Range
350–450 bp (400-base-read library)	8 minutes	5–12 minutes
270–370 bp (300-base-read library)	10 minutes	5–15 minutes
200–300 bp (200-base-read library)	15 minutes	5–30 minutes
150–250 bp (150-base-read library)	20 minutes	10–40 minutes
100–200 bp (100-base-read library)	40 minutes	30–60 minutes

7. Add 5 µL of Ion Shear™ Stop Buffer immediately after incubation, and mix thoroughly by vortexing for at least 5 seconds. Store the reaction tube on ice.

Purify the fragmented DNA

IMPORTANT! Use freshly prepared 70% ethanol (1 mL plus overage per sample) 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 99 µL of Agencourt™ AMPure™ XP Reagent (1.8X sample volume) to the sheared DNA sample, pipet up and down five times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate the mixture at room temperature for 5 minutes.

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 of brown tint when viewed at an angle. Carefully remove and discard the supernatant without disturbing the bead 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 25 µL of Low TE directly to the pellet to disperse the beads. Pipet the suspension up and down five times, then vortex the sample for 10 seconds to mix.

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 0.2-mL PCR tube without disturbing the pellet.

   IMPORTANT! The supernatant contains your sample. **Do not discard.**

10. *(Optional)* Check the fragment size using 1 µL of eluted DNA and the Agilent™ 2100 Bioanalyzer™ instrument and Agilent™ High Sensitivity DNA Kit. Confirm the desired DNA fragment size range as follows:

Sequencing System	Library Type	Target Median Fragment Size	Fragment Size Range
Ion PGM™ System or Ion S5™/Ion S5™ XL System	400-base-read library	350–450 bp	150–1000 bp
	300-base-read library	270–370 bp	100–900 bp
	200-base-read library	200–300 bp	100–700 bp
	100-base-read library	100–200 bp	50–500 bp
Ion Proton™ System	200-base-read library	150–250 bp	100–700 bp
	150-base-read library	100–200 bp	50–500 bp

11. Proceed to “Ligate adaptors, nick repair, and purify“ on page 16.

Ligate adaptors, nick repair, and purify

Materials required

Materials required from the Ion Plus Fragment Library Kit

- 10X Ligase Buffer
- DNA Ligase
- Nick Repair Polymerase
- dNTP Mix
- Low TE

Materials required from the Ion Xpress™ Barcode Adapters Kits (for barcoded libraries)

- Ion Xpress™ P1 Adapter
- Ion Xpress™ Barcode X (1 barcode adapter per sample)

Other materials and equipment

- 0.2-mL PCR tubes
- Thermal cycler
- Nuclease-free Water
- Agencourt™ AMPure™ XP Reagent
- 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 Agencourt™ 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. 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.

4. Repeat step 3 for a second wash.

5. 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.

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

7. 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.

8. 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.

9. Proceed to "Size-select the unamplified library" on page 19.

Size-select the unamplified library

This section describes two options for size-selection:

- "Option 1: Size-select with E-Gel™ SizeSelect™ Agarose Gels" on page 19
- "Option 2: Size-select with the Pippin Prep™ instrument" on page 24

For each method, target the peak length of the size-selected library according to the desired read length:

Sequencing System	Library Size	Target Peak Size
Ion PGM™ System, or Ion S5™/Ion S5™ XL System	400-base-read	~480 bp
	300-base-read	~390 bp
	200-base-read	~330 bp
	100-base-read	~200 bp
Ion Proton™ System	200-base read	~270 bp
	150-base-read	~220 bp

Visit the Ion Community at **ioncommunity.thermofisher.com** for other library size-selection methods.

Option 1: Size-select with E-Gel™ SizeSelect™ Agarose Gels

Materials required

Materials provided in the Ion Plus Fragment Kit

- Low TE

Other materials and equipment

- E-Gel™ iBase™ unit and E-Gel™ Safe Imager™ transilluminator combo kit
- E-Gel™ SizeSelect™ 2% Agarose Gel
- **100-, 150-, 200- or 400-base-read libraries:** 50-bp DNA Ladder (Cat. No. 10416-014; do not substitute other 50-bp ladders such as the TrackIt™ 50-bp Ladder)
- **300-base-read libraries:** 100-bp DNA Ladder (Cat. No. 15628-019; do not substitute other 100-bp ladders such as the TrackIt™ 100-bp Ladder)
- Nuclease-free Water

Prepare, load, and run the gel

Start with unamplified library, non-barcoded or barcoded, prepared and purified as described in "Ligate adaptors, nick repair, and purify" on page 16.

IMPORTANT! We recommend that first-time users of the E-Gel™ SizeSelect™ 2% Agarose Gel refer to the *E-Gel™ Technical Guide* and *E-Gel™ SizeSelect™ Agarose Gels Quick Reference,* available at **ioncommunity.thermofisher.com**

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1. Prepare the E-Gel™ iBase™ unit and E-Gel™ Safe Imager™ transilluminator combo unit:
   a. Place the iBase™ unit on top of the Safe Imager™ transilluminator, and plug the short cord from the Safe Imager™ into the power inlet of the iBase™ unit.
   b. Plug the connector of the power cord with the transformer into the Safe Imager™ transilluminator and connect the other end of the power cord to an electrical outlet.
   c. Verify that the iBase™ has the "SizeSelect™ 2%" program. If not, refer to "Downloading upgrade" from the *E-Gel™ Technical Guide.*
2. Load an E-Gel™ SizeSelect™ 2% Agarose gel in the E-Gel™ iBase™ unit:
   a. Remove the gel from the package and gently remove the combs from the SizeSelect™ cassette.
   b. Insert the gel cassette into the E-Gel™ iBase™ unit right edge first.
   c. Press firmly at the left edge of the cassette to seat the gel in the base. A steady light illuminates on the iBase™ unit when the cassette is properly inserted.
3. Load the gel without pre-running, using the following guidelines for the most accurate size cuts:
   - Load no more than 250 ng of the appropriate DNA Ladder (50-bp DNA Ladder for 100-, 150-, 200-, or 400-base-read libraries, 100-bp DNA Ladder for 300-base-read libraries).
   - Do not pierce the agarose at the bottom of the wells of the gel.
   - Do not use wells #1 and #8 at either edge of the gel (the edge effect slows the sample migration, resulting in shorter fragments), and do not use the wells right next to the ladder well in the center (to avoid potential cross contamination with the ladders).
   - Do not load different libraries in adjacent wells, to avoid potential cross contamination.

   a. Add 20 µL of ligated DNA to the loading well (top row).
   b. Dilute the appropriate 1 µg/µL DNA Ladder in Low TE buffer to 25 ng/µL (1:40 dilution). Add 10 µL of diluted DNA ladder into the middle well, lane M. Load no more than 250 ng (10 µL of 1:40 dilution) of the DNA Ladder.
   c. Add 25 µL of Nuclease-free Water to all empty wells in the top row.

d. Add 25 µL of Nuclease-free Water to all the large wells in bottom row (collection wells), and add 10 µL to the center well (lane M) of the bottom row.

4. Run the E-Gel™ SizeSelect™ 2% Agarose Gel:

a. Place the amber filter over the E-Gel™ iBase™ unit.

b. Select **Run SizeSelect 2%** program, and set the time to the value under **Run Time to Reference Line** in the Run Time Estimation Table in the *E-Gel™ SizeSelect™ Agarose Gels Quick Reference* for the appropriate band size, as described below. If you are a new user, select the shorter run time.

Sequencing System	Library Size	Target Library Length	Run Time to Reference Line
Ion PGM™ System or Ion S5™/Ion S5™ XL System	400-base-read	480 bp	16–20 minutes
	300-base-read	390 bp	15–17 minutes
	200-base-read	330 bp	12–14 minutes
	100-base-read	200 bp	11–12.5 minutes
Ion Proton™ System	200-base-read	270 bp	11–14 minutes
	150-base-read	220 bp	11–14.5 minutes

c. Press **Go** on the iBase™ unit to start electrophoresis. The red light turns to green.

d. Monitor the appropriately sized ladder band to the reference line with periodic monitoring of the run. If needed, extend the run time by repeating steps 4.b to 4.d with very short run time settings.

e. Press **Go** again to stop the run when the band reaches the reference line.

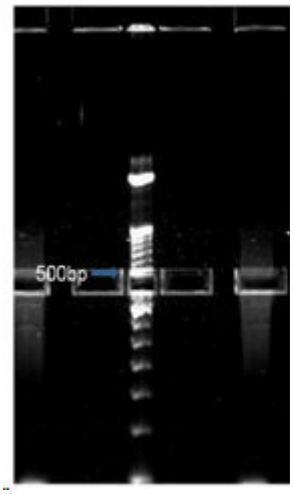
f. Refill the collection wells to 25 µL with ~10 µL of Nuclease-free Water. The water in the wells should form a concave surface. **Do not overfill.**

g. Repeat steps 4b to 4d with the run time set to 0.5–1.5 minutes, the value under **Run Time from Reference Line to Collection Well** in the Run Time Estimation Table in the *E-Gel™ SizeSelect™ Agarose Gels Quick Reference*.

h. Monitor the middle marker well (M) frequently for the desired fragment length, and stop the run when the desired fragment size range is in the collection well as shown in the following figures.

400-base-read library (480-bp target peak):

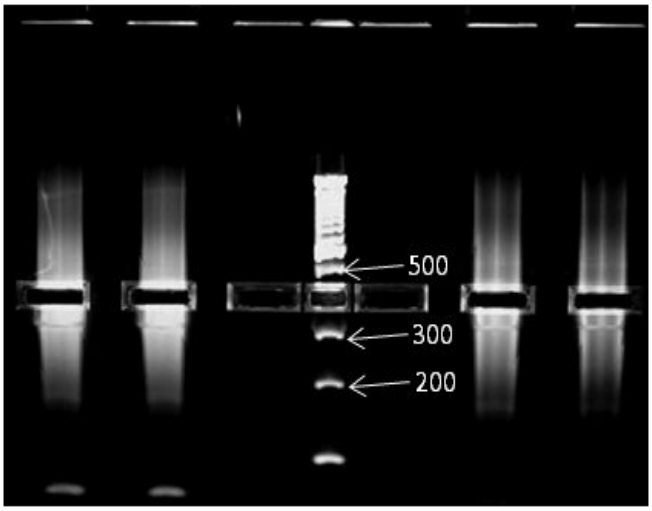
Stop the run when the 500-bp ladder band is at the top edge of the collection well.



400-base-read library gel

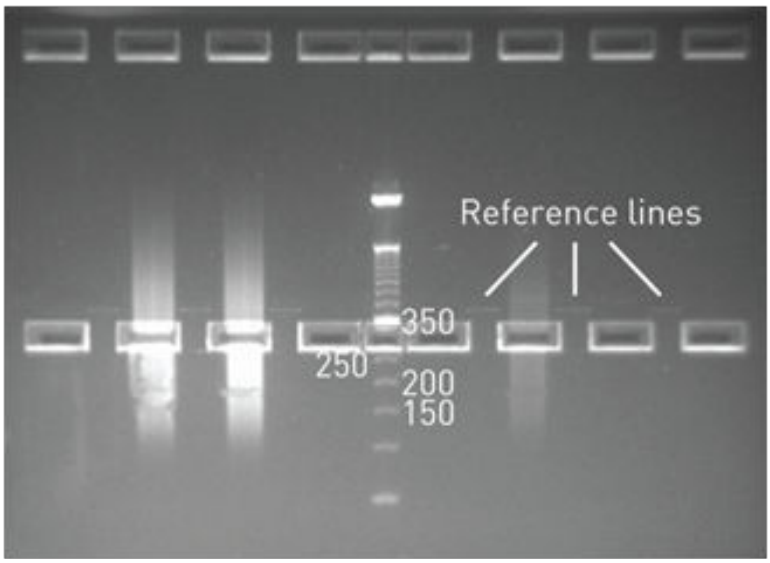
300-base-read library (390-bp target peak):

Stop the run when the 400-bp ladder band is in the middle of the collection well or the 500-bp ladder band is aligned with the reference line.



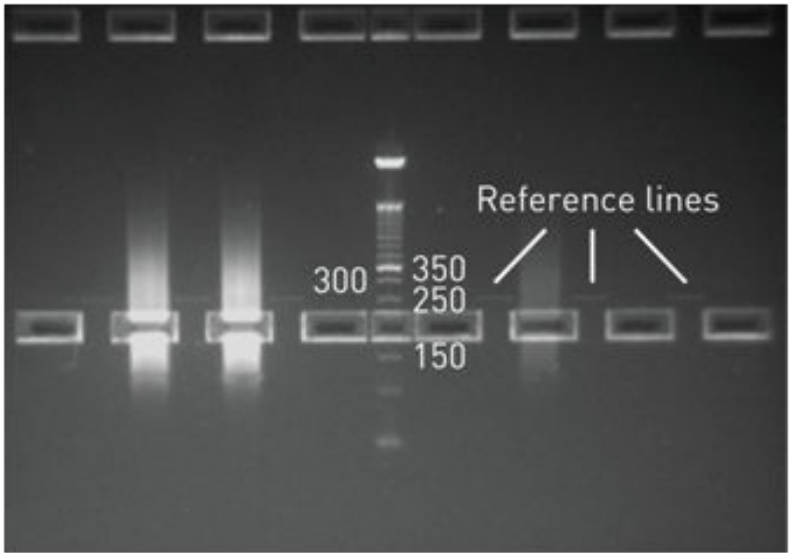
300-base-read library gel

200-base-read library (330-bp target peak):
Stop the run when the 350-bp ladder band has just completely entered the top edge of the collection well.



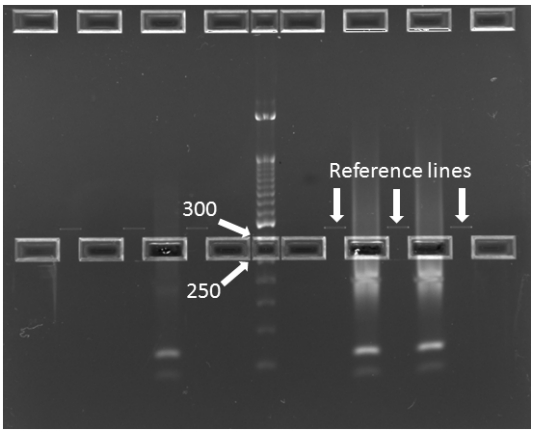
200-base-read library gel

100–150-base-read libraries (200–220-bp target peak):
Stop the run when the 200-bp ladder band is in the middle of the collection well.



100-base-read library gel

200-base-read Ion Proton™ libraries (270-bp target peak):
Stop the run when the 250-bp band re-emerges from the collection well as 300-bp band begins to enter the well.



200-base-read Ion Proton™ library gel

5. Collect the sample:
   a. Collect the solution from the collection wells using a pipette, without piercing the bottom of the well.
   b. Refill the well with 10 µL Nuclease-free Water to wash the collection well, collect the solution, and pool the solutions. The total recovered volume is ~30 µL from each well.
   c. Dispose of the used gels as hazardous waste.
6. Proceed to "Determine if library amplification is required" on page 28.

Option 2: Size-select with the Pippin Prep™ instrument

Materials required

Materials provided in the kit

- Low TE

Other materials and equipment

- Pippin Prep™ instrument (Cat. No. 4471271)
- 2% Agarose Gel Cassettes for the Pippin Prep™ instrument; includes Loading Solution, Marker B, and Electrophoresis Buffer (Cat. No. 4472170)
- Nuclease-free Water
- Agencourt™ AMPure™ XP Kit
- Freshly prepared 70% ethanol
- Magnetic rack

Set up the instrument and perform the run

Start with unamplified library, non-barcoded or barcoded, prepared and purified as described in "Ligate adaptors, nick repair, and purify" on page 16.

IMPORTANT! The protocol below closely follows the Pippin Prep™ instrument manual. Novice users may want to review training videos at **http://www.sagescience.com/resources** before using the instrument for the first time. Software version 3.71 or higher versions are required to turn off signal monitoring of the sample lanes.

---

1. Define plate layout and separation parameters on the Protocol Editor screen.

   IMPORTANT! For consistent results, before each run calibrate the optics with the calibration fixture. Place the calibration fixture onto the optical nest. Close the lid and press **CALIBRATE** to launch the calibration window. Enter 0.80 in the "Target I ph, mA" field. Press the **CALIBRATE** button in the window, and when complete, press **EXIT**.

   - For 100–300-base-read libraries:
     a. From the cassette type drop-down menu, choose **2% Marker B No Overflow Detection**.
     b. Select the "Tight" collection mode for each lane and then define the BP Target setting for each of 1–4 lanes used.

Sequencing System	Library Size	BP Target Setting
Ion PGM™ System or Ion S5™/Ion S5™ XL System	300-base-read	390 bp
	200-base-read	315 bp
	100-base-read	180 bp
Ion Proton™ System	200-base-read	270 bp
	150-base-read	220 bp

     c. Define lanes 1–4 as sample lanes and 5 as the ladder lane by entering "5" in the reference lane box and selecting the "Apply Reference to all Lanes" button. Ensure that the "Ref Lane" value for each lane is 5.
     d. Set the run time for 1.5 hours.
   - For 400-base-read libraries:
     a. From the cassette type drop-down menu, choose **2% DF Marker L**.
     b. Select the "Tight" collection mode for each lane and then define the BP Target setting for each lane as 475.
     c. Define lanes 1–5 as sample lanes and press the **Use Internal Standards** button to match the lane numbers and ensure that the "Ref Lane" values match the lane numbers.
     d. Set the run time for 1.5 hours.

2. Prepare the 2% Agarose Gel cassette for the Pippin Prep™ instrument:
   a. Unwrap the cassette, and then tip it toward the loading wells end to dislodge any air bubbles present around the elution wells and then insert the cassette into the instrument.

b. Remove the two adhesive strips covering the loading wells and elution wells.

c. Fill the loading wells with Electrophoresis Buffer to the top so that a concave meniscus forms.

d. Remove all liquid from the elution wells, and then add 40 µL of Electrophoresis Buffer.

e. Seal the elution wells with the adhesive tape strips supplied with the cassette packaging.

f. Following the Pippin Prep™ manual, apply current across the cassette and confirm that the current across both the separation ports and the elution ports is within specifications.

3. Load the sample:

IMPORTANT! Do not pierce the agarose at the bottom of the wells of the gel.

- For 100–300-base-read libraries:
  a. Add 10 µL of Low TE to the purified ligated DNA (20 µL) to bring the volume to 30 µL.
  b. Add 10 µL of Loading Solution. The total volume is 40 µL for each sample.
  c. Go to the Main screen, then choose the newly generated separation file (or a previously saved file) from the Protocol Name pull-down menu.
  d. Remove 40 µL of Electrophoresis Buffer from the loading well of the designated Ref Lane, then load 40 µL of 2% DNA Marker B.
  e. Remove 40 µL of Electrophoresis Buffer from one sample loading well at a time, then immediately load the entire 40-µL sample into the well.

  IMPORTANT! Load the sample immediately to minimize buffer re-entering the well. Buffer in the well prevents loading the entire sample.

- For 400-base-read libraries:
  a. Add 10 µL of Low TE to the purified ligated DNA (20 µL) to bring the volume to 30 µL.
  b. Add 10 µL of Loading Solution/marker mix (labeled Marker L). The total volume is 40 µL for each sample.
  c. Go to the Main screen, then choose the newly generated separation file (or a previously saved file) from the Protocol Name pull-down menu.
  d. Remove 40 µL of Electrophoresis Buffer from one sample loading well at a time, then immediately load the entire 40-µL sample into the well.

  IMPORTANT! Load the sample immediately to minimize buffer re-entering the well. Buffer in the well prevents loading the entire sample.

4. Run the instrument:
   a. When the ladder and all samples are loaded, close the lid of the Pippin Prep™ instrument.

   b. On the Main screen, press **Start** to initiate the run.

c. When the separation is complete, transfer the DNA from the elution wells (typically 40–60 µL) with a pipet to new 1.5-mL Eppendorf LoBind™ Tubes.

d. Add Nuclease-free Water to the DNA to bring the volume to 60 µL.

Purify the size-selected DNA

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

1. Add 108 µL of Agencourt™ AMPure™ beads (1.8X sample volume) to the sample, pipet up and down five times to mix the bead suspension thoroughly with the DNA, pulse-spin the tube, then incubate the mixture for 5 minutes at room temperature.
2. Pulse-spin, then place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.
3. Without removing the tube from the magnet, add 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, then 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 ≤5 minutes.
8. Remove the tube from the magnetic rack, then add 25 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, then 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.**

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 29
No	"Qualify non-barcoded libraries" on page 31 *or* "Qualify and pool barcoded libraries" on page 33

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. 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 (size-selected by E-Gel™ SizeSelect™ Agarose Gels or Pippin Prep™ instrument)	25 µL
Total	**130 µL**

2. Split the 130-µL reaction into two 0.2-mL PCR tubes, each containing about 65 µL.

3. 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 (8 cycles)	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	70°C	1 min
Hold	—	4°C	Hold for up to 1 h

4. 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 31.
    For barcoded libraries, proceed to "Qualify and pool barcoded libraries" on page 33.

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 31.

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 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**.

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Revision history: Pub. No. MAN0007044

Revision	Date	Description of Change
B.0	28 April 2016	• 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**

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28 April 2016