USER GUIDE

Ion Xpress™ Plus gDNA Fragment Library Preparation

for use with:
Ion Xpress™ Plus Fragment Library Kit
Ion Plus Fragment Library Kit
Ion Xpress™ Barcode Adapters 1-96 Kit
Ion Plus Fragment Library Adapters
Ion Library Equalizer™ Kit

Catalog Number 4471269, 4471252, 4476340, 4471250, 4474009, 4474518, 4474519, 4474520, 4474521, and 4482298

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About this guide

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

Revision history

Revision	Date	Description of Change
C.0	29 April 2014	• Instructions for splitting PCR tubes clarified in Chapter 7. • Purification protocol for E-Gel®-size-selected libraries added back to Appendix A.
B.0	3 March 2014	• Changed the recommendation for final library dilution to 100 pM to unify the kit with other Ion library and template kits.
A.0	6 December 2013	• Tech Access release. • Publication number changed from 4471989 to MAN0009847 and version numbering reset to A.0 in conformance with internal document control.
N	20 June 2013	• Updated for 200-base-read libraries for Ion Proton™ System sequencing.
M	15 May 2013	• Modified the "Elute the equalized library" protocol in Appendix A.
L	6 March 2013	• Added new shearing and size-selection methods for 400-base-read libraries. • Note that Equalizer™ is only appropriate for up to 300-base-read libraries
K	11 February 2013	• Added Ion Library Equalizer™ Kit protocol in new Appendix A
J	23 January 2013	• Updated support for the Ion Proton™ System. • Updated Covaris® protocol in Appendix A.
H	11 January 2013	• Internal release.
G	21 December 2012	• Internal release.

Revision	Date	Description of Change
F	28 September 2012	• Internal release.
E	10 September 2012	• Added support for 300-base-read libraries.

Library preparation methods covered by this guide

The procedures in this guide are compatible with the Ion Xpress™ Plus Fragment Library Kit (Cat. no. 4471269), the Ion Plus Fragment Library Kit (Cat. no. 4471252), Ion Xpress™ Barcode Adapters 1–96 (various catalog numbers), and the Ion Library Equalizer™ Kit (Cat. no. 4482298).

For amplicon library preparation using the Ion Xpress™ Plus or Ion Plus Fragment Library Kit, see the user bulletins *Prepare Amplicon Libraries without Fragmentation Using the Ion Plus Fragment Library Kit* (Pub. no. MAN0006846) and *Prepare Amplicon Libraries Requiring Fragmentation Using the Ion Xpress™ Plus Fragment Library Kit* (Pub. no. MAN0007044).

1 Product information

Product description

Library kits

Use one or both of the following kits to prepare fragment libraries from genomic DNA (gDNA) for downstream template preparation and sequencing on the Ion Personal Genome Machine® (PGM™) System or the Ion Proton™ System:

- Ion Plus Fragment Library Kit (Cat. no. 4471252): This kit includes reagents for end-repair of physically fragmented gDNA and reagents for library preparation from the end-repaired DNA.
- Ion Xpress™ Plus Fragment Library Kit (Cat. no. 4471269): This kit includes Ion Shear™ Plus Reagents for enzymatic fragmentation of gDNA plus the contents of the Ion Plus Fragment Library Kit (see above) for library preparation from the enzymatically fragmented DNA.

Barcode Adapters

Ion Xpress™ Barcode Adapters kits include the P1 adapter and barcoded A adapters that substitute for the non-barcoded adapter mix supplied in the Ion Plus Fragment Library Kit. Barcoded library preparation is otherwise identical to non-barcoded library preparation.

Ion Library Equalizer™ Kit

The Ion Library Equalizer™ Kit provides an optional, streamlined method for normalizing library concentration without the need for quantification, for up to 300-base-read libraries. The equalized library can be used directly in template preparation.

Ion Plus Fragment Library Adapters

The Ion Plus Fragment Library Adapters Kit (Cat. no. 4476340) contains additional adapters and Library Amplification Primer Mix for preparing ≤20 libraries at 100 ng input, and ≤10 libraries at 1 µg input. The adapters can be used with the library kits listed above.

Template kit compatibility

These library kits are compatible with all current Ion template preparation kits for the Ion PGM™ System and Ion Proton™ System.

Kit contents and storage

Ion Plus Fragment Library Kit

The Ion Plus Fragment Library Kit (Cat. no. 4471252) contains one box. 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:

Ion Plus Fragment Library Kit (Cat. no. 4471252)				
Component	**Cap Color**	**Quantity**	**Volume**	**Storage**
5X End Repair Buffer	Red	1 tube	400 µL	–30°C to –10°C
End Repair Enzyme[1]	Orange	1 tube	20 µL	
10X Ligase Buffer	Yellow	1 tube	200 µL	
DNA Ligase	Blue	1 tube	40 µL	
Nick Repair Polymerase	Clear	1 tube	160 µL	
dNTP Mix	Violet	1 tube	40 µL	
Adapters	Green	1 tube	100 µL	
Platinum®PCR SuperMix High Fidelity	Black	2 tubes	1 mL each	
Library Amplification Primer Mix	White	1 tube	100 µL	
Low TE	Clear	2 tubes	1.25 mL each	Room temperature (15°C to 30°C) *or* –30°C to –10°C

[1] 5X End Repair Buffer and End Repair Enzyme are required only for physically fragmented gDNA.

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 (listed below) and the Ion Plus Fragment Library Kit (see contents above).

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:

Ion Xpress™ Plus Fragment Library Kit (Cat. no. 4471269)				
Ion Shear™ Plus Reagents Kit				
Component	**Cap Color**	**Quantity**	**Volume**	**Storage**
Ion Shear™ Plus 10X Reaction Buffer	Clear	2 tubes	50 µL each	–30°C to –10°C
Ion Shear™ Plus Enzyme Mix II [1]	Clear	2 tubes	100 µL each	
Ion Shear™ Plus Stop Buffer	Clear	2 tubes	50 µL each	
Ion Plus Fragment Library Kit (see components listed in previous table)				

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

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)

And the complete set of adapters:

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

Each barcode kit is sufficient for preparing ≤10 libraries per barcode (10 × 16 libraries) for 100 ng input, or 2 libraries per barcode for 1 µg input, and contains the following components:

Component	**Cap Color/ Label**	**Quantity**	**Volume**	**Storage**
Ion Xpress™ P1 Adapter	Violet/—	1 tube	320 µL	–30°C to –10°C
Ion Xpress™ Barcode X[1]	White/X	16 tubes (one per barcode)	20 µL each	

[1] X = 1 through 16

Ion Plus Fragment Library Adapters

The Ion Plus Fragment Library Adapters Kit (Cat. no. 4476340) contains additional adapters and Library Amplification Primer Mix for preparing ≤20 libraries at 100 ng input, and ≤10 libraries at 1 µg input. The kit contains the following components:

Component	Cap Color	Quantity	Volume	Storage
Adapters	Green	1 tube	100 µL	–30°C to –10°C
Library Amplification Primer Mix	White	1 tube	100 µL	

Ion Library Equalizer™ Kit

The Ion Library Equalizer™ Kit (Cat. no. 4482298) contains reagents for 96 reactions:

Component	Cap Color	Quantity	Volume	Storage[1]
Equalizer™ Primers	Red	1 tube	200 µL	2°C to 8°C
Equalizer™ Capture Solution	Purple	1 tube	1 mL	
Equalizer™ Elution Buffer	—	1 bottle	10 mL	
Equalizer™ Beads	Orange	1 tube	300 µL	
Equalizer™ Wash Buffer	—	1 tube	35 mL	Room temp (15°C to 30°C)

[1] The kit is shipped at ambient temperature. Store as indicated.

Required materials and equipment

Required for all types of library peparation

Use the Agencourt® AMPure® XP Kit for DNA purification. Use the Agilent® 2100 Bioanalyzer® instrument to analyze DNA fragment length distribution during library preparation.

Description	Supplier	Cat. no.	Quantity
Agencourt® AMPure® XP Kit	Beckman Coulter	A63880 or A63881	1 kit
DynaMag™-2 magnet (magnetic rack)	Life Technologies	12321D	1 rack
Agilent® 2100 Bioanalyzer® instrument	Agilent	G2939AA	1 instrument
Agilent® High Sensitivity DNA Kit	Agilent	5067-4626	1 kit
1.5-mL Eppendorf LoBind® Tubes	Eppendorf	022431021	1 box

Description	Supplier	Cat. no.	Quantity
0.2-mL PCR tubes	MLS[1]	—	—
Microcentrifuge	MLS	—	1 microcentrifuge
Thermal cycler	MLS	—	1 thermal cycler
Vortex mixer	MLS	—	1 vortex mixer
Pipettors 1–1000 µL	MLS	—	1 each
Barrier pipette tips	MLS	—	1 box each
Nuclease-free Water	Life Technologies	AM9932	1000 mL
Optional: Ion Library Quantitation Kit (required for quantitation of unamplified libraries)	Life Technologies	4468802	1 kit
Optional: Ion PGM™ Controls 200 Kit *or* Ion PI™ Controls Kit v2 *or* Ion PI™ Controls 200 Kit	Life Technologies	4480449 *or* 4482414 *or* 4488985	1 kit
Optional: 10 mM Tris, pH 7.5–8.5	MLS	—	—
Optional: RNase I (100 units/µL)	Life Technologies	AM2294 AM2295	10,000 units 25,000 units
Optional: PureLink® Genomic DNA Kit (for cleanup after optional RNase treatment)	Life Technologies	K1820-01 K1820-02 K1820-03	50 preps 250 preps 4 × 96-well plates

[1] Major Laboratory Supplier.

Required for physical fragmentation of gDNA

For physical fragmentation of gDNA, use any of the systems listed in the following table:

Description	Supplier	Cat. no.	Quantity
Bioruptor® Standard Sonication System with accessories for 12 x 0.5 mL tubes (Microtube Attachment & Gearplate) *or*	Life Technologies *or* Diagenode	4465622 or UCD-200 TS	1 system
Bioruptor® NGS Sonication System with accessories for 12 x 0.5 mL tubes (Microtube Attachment & Gearplate) [1]	Diagenode	UCD-600 TS	1 system

Description	Supplier	Cat. no.	Quantity
Bioruptor® 0.5-mL Microtubes for DNA Shearing	Life Technologies *or* Diagenode	4465623 or C30010013	500 tubes
Covaris® M220 System (110 V for U.S. customers; 220 V for international customers)	Covaris	—	1 system
Covaris® AFA-grade Water[2]	Covaris	—	—
Low TE (10 mM Tris pH 8.0, 0.1 mM EDTA)	MLS	—	—
Covaris®S2 System (110 V for U.S. customers; 220 V for international customers) *or* Covaris® S220 System (110 V for U.S. customers; 220 V for international customers)	Covaris	—	1 system
Covaris® microTUBE™ tubes	Covaris	—	25 snap-cap tubes

[1] The Bioruptor® NGS Sonication System is discontinued and has been replaced by the Bioruptor® Pico Sonication System (Thermo Fisher Scientific Cat. no. 4486162 or Diagenode Cat. no. B0106001).

[2] Highly purified water (≥ASTM Type III or ISO Grade 3) may also be used.

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

Description	Supplier	Cat. no.	Quantity
E-Gel® iBase™ unit and E-Gel® Safe Imager™transilluminator combo kit	Life Technologies	G6465	1 unit and kit
E-Gel® SizeSelect™ 2% Agarose	Life Technologies	G6610-02	10/pack
50-bp DNA ladder (1 µg/µL), required for 100-, 150-, 200- and 400-base-read libraries	Life Technologies	10416-014	1 ladder
100-bp DNA ladder (1 µg/µL), required for 300-base-read libraries	Life Technologies	15628-019	1 ladder

Required for size-selection using the Pippin Prep™ instrument

Description	Supplier	Cat. no.	Quantity
Pippin Prep™ instrument	Life Technologies	4471271	1 instrument
2% Agarose Gel Cassettes for the Pippin Prep™ instrument (for 100–300-base-read libraries)	Life Technologies	4472170	10 cassettes
2% Dye Free Marker L Agarose Gel Cassettes for the Pippin Prep™ instrument (for 400-base-read libraries)	Sage Science	CDF2010	10 cassettes

2 Procedure guidelines and overview

Procedure guidelines

- **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 Appendix C, "Evaluate the quality of the genomic DNA" for more information about assessing the integrity and size of your input DNA material and performing an optional RNase treatment procedure.
- First-time users may prepare a library to familiarize themselves with the fragmentation and library preparation procedures prior to using their own samples. To prepare a genomic fragment library using the Ion Shear™ Plus Reagents, use 100 ng (1 µL) of the *E. coli* DH10B Control DNA supplied in the Ion PGM™ Controls Kit (Cat. no. 4480449) for Ion PGM™ System sequencing, or 100 ng (1 µL) of Human CEPH Genomic DNA Control supplied in the Ion PI™ Controls Kit v2 (Cat. no. 4482414) or Ion PI™ Controls 200 Kit (Cat. no. 4488985) for Ion Proton™ System sequencing. Use 100 ng or 1 µg (1 µL or 10 µL, respectively) of the appropriate DNA to prepare a genomic fragment library using the Bioruptor® Sonication System.
- 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 (Eppendorf® Part no. 022431021).
- 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.

Procedure overview

First, gDNA is fragmented to appropriately sized, blunt-ended DNA fragments. The fragment DNA is ligated to Ion-compatible adapters, followed by nick-repair to complete the linkage between adapters and DNA inserts. For barcoded libraries, substitute adapters from the Ion Xpress™ Barcode Adapters kits.

The adapter-ligated library is then size-selected for optimum length according to target read length as shown in the following tables:

Library sizes for Ion PGM™ System sequencing

Target Read Length	Median Insert Size	Median Library Size
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

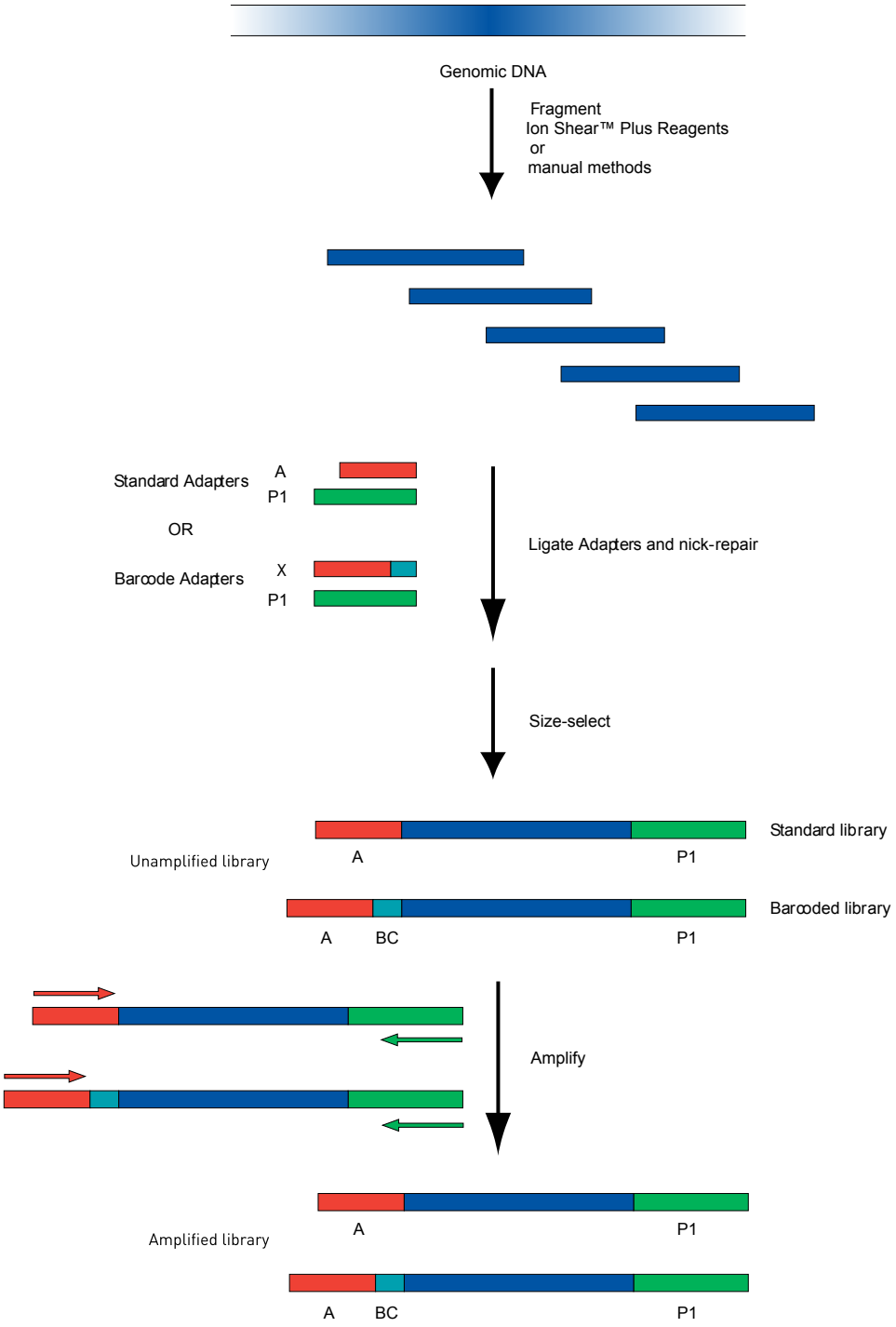
Target Read Length	Median Insert Size	Median Library Size
200 bases (200-base-read library)	~200 bp	~270 bp
150 bases (150-base-read library)	~150 bp	~220 bp

Final amplification of the library is optional, depending on the amount of input DNA and your experimental requirements.

You may also use the Ion Library Equalizer™ Kit to eliminate the need for library quantification and dilution for up to 300-base-read libraries. Libraries are then ready for template preparation on Ion Sphere™ Particles using an appropriate Ion template preparation kit.

Workflow diagram

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.



Workflow options

Choose the workflow options based on your experimental needs and lab setup.

Fragmentation and end-repair options to prepare adapter-compatible DNA; 30–120 min		
Method	Ion Shear™ Plus Reagents (no end-repair)	Bioruptor® System fragmentation + end-repair
Input amount	50–100 ng or 1 µg gDNA	100 ng or 1 µg gDNA
Library Size[1]	100–400-base-read libraries	100–400-base-read libraries
Features	Fragmentation profile tuned by reaction time—no end-repair	Same fragmentation conditions for 200-base-read and 100-base-read libraries using the Bioruptor® NGS UCD-600
Time	Ion PGM™ System: 300-base-read and above libraries: ~30 min 200-base-read libraries: ~40 min 100-base-read libraries: ~60 min	55–120 min depending on the library and instrument used
	Ion Proton™ System: 200-base-read libraries: ~50 min 150-base-read libraries: ~60 min	

[1] Includes 150- and 200-base-read libraries for the Ion Proton™ System. Sequence other library sizes on the Ion PGM™ System.

▼

Adapter ligation and nick repair; ~40 min

▼

Size-selection options; 20-90 min		
Method	E-Gel® SizeSelect™ 2% Agarose	Pippin Prep™ instrument
Features	• Quicker; Broader size distribution	• Automated; Tighter size distribution results in more consistent library size
Time	400-base-read libraries: ~35 min 300-base-read libraries: ~30 min 100–200-base-read libraries: ~20 min	90 min

▼

Library normalization/quantification options[1]; 40–70 min			
Method	Ion Library Equalizer™ Kit	qPCR	Bioanalyzer® analysis
Features	No library quantification required	Amplification optional[1]	Amplification optional[1]
Time	~40 minutes	~70 minutes	~60 min

[1] Library amplification is recommended for Ion Proton™ System sequencing.

3 Prepare adapter-compatible DNA

This section describes two methods for preparing adapter-compatible DNA, as appropriate for your input DNA and desired library size:

Method	Library Size[1]	Input gDNA	Starting on...
Ion Shear™ Plus Reagents	100–400-base-read	50–100 ng or 1 µg	"Method 1: Fragment gDNA with Ion Shear™ Plus Reagents" on page 20
Bioruptor® System + end-repair	100–400-base-read	100 ng or 1 µg	"Method 2: Fragment gDNA with the Bioruptor® Sonication System" on page 24

[1] Includes 150- and 200-base-read libraries for the Ion Proton™ System. Sequence other library sizes on the Ion PGM™ System.

Note: For alternative manual fragmentation methods, refer to Appendix B, "Fragment DNA with the Covaris System". Up to 400-base-read libraries may be prepared with the Covaris® M220 sonicator and the Covaris® S2 and S220 Systems.

Method 1: Fragment gDNA with Ion Shear™ Plus Reagents

This section describes conditions for enzymatic fragmentation of gDNA into blunt-ended fragments. This method is suitable for 100–400-base-read libraries including the 150- and 200-base-read libraries for the Ion Proton™ System.

The fragmented DNA is ready for adapter ligation. No end-repair is required.

Genomic DNA

Prepare high-quality, RNA-free genomic DNA (gDNA) using any of a number of commercially available kits. Prepare 50–100 ng or 1 µg gDNA per library.

Note: For the Ion Proton™ System, use a minimum of 1 µg of DNA for unamplified libraries.

Note: The Ion Shear™ reaction is optimized for high-quality gDNA. If you want to use the Ion Shear™ Reagents with DNA from formalin-fixed, paraffin-embedded tissue (FFPE DNA), you must determine optimum reaction conditions. We suggest trying a lower reaction temperature and a shorter reaction time to achieve the desired fragment size.

Note: The Ion Shear™ reaction has very good tolerance of 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. For 1 µg-input samples, it

is good practice to confirm that the reaction time is optimal for your laboratory conditions.

Library and fragment sizes

The Ion Shear™ fragmentation method is suitable for 100-400-base-read libraries. Choose the fragmentation conditions according to the desired library size.

Sequencing System	Library Size	Median Fragment Size
Ion PGM™ System	400-base-read	350–450 bp
	300-base-read	270–370 bp
	200-base-read	200–300 bp
	100-base-read	100–200 bp
Ion Proton™ System	200-base-read	150–250 bp
	150-base-read	100–200 bp

Fragmentation and purification procedure

Materials 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 ***or*** Human CEPH DNA Control

Fragment the DNA

IMPORTANT! 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.

Note: *(Optional)* Prepare a control sample in a separate tube. Use 1 µL (100 ng) of *E. coli* DH10B Control DNA for the Ion PGM™ System or 1 µL (100 ng) of Human

CEPH DNA Control for the Ion Proton™ System, mixed with 9 µL of Nuclease-free Water or 10 mM Tris, pH 7.5–8.5.

1. Determine the volume of input gDNA, and adjust the concentration as necessary.
   - For 1 µg input: Prepare 10 µL at 100 ng/µL in Nuclease-free Water or 10 mM Tris, pH 7.5–8.5.
   - For 50–100 ng input: Determine the volume containing 50–100 ng. If dilution of the DNA sample is necessary, use Nuclease-free Water or 10 mM Tris, pH 7.5–8.5 as diluent.

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 by Input DNA	
	50-100 ng	1 µg
gDNA, 50-100 ng	γ µL	—
gDNA, 100 ng/µL	—	10 µL
Ion Shear™ Plus 10X Reaction Buffer	5 µL	5 µL
Nuclease-free Water	35 – γ µL	25 µL
Total	**40 µL**	**40 µL**

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

5. Using a P100–P200 pipettor set at a 40-µL volume, mix the reaction by rapidly pipetting up and down 8-10 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.

   Note: 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	8 minutes	5–12 minutes
270–370 bp	10 minutes	5–15 minutes
200–300 bp	15 minutes	5–30 minutes
150–250 bp	20 minutes	10–40 minutes
100–200 bp	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 5 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. 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 magnet, air-dry the beads at room temperature for ≤5 minutes.

7. Remove the tube from the magnetic rack, and add 25 µL of Low TE directly to the pellet to disperse the beads. Mix thoroughly by pipetting the suspension up and down 5 times, then vortex the sample for 10 seconds.

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 0.2-mL PCR tube without disturbing the pellet.

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

9. *(Optional)* Check the fragment size using the indicated volume of the eluted DNA and the Agilent® 2100 Bioanalyzer® instrument and Agilent® High Sensitivity DNA Kit.

Volume by Input DNA	
50–100 ng	**1 µg**
1 µL	1 µL of 1:10 dilution[1]

[1] Prepare the dilution in Nuclease-free Water

Confirm the desired DNA fragment size range as follows:

Sequencing System	Library Type	Target Median Fragment Size	Fragment Size Range
Ion PGM™ 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

See Figures 1–2 in Appendix D, "Bioanalyzer® instrument analysis" for example traces.

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

10. Proceed to Chapter 4, "Ligate adapters, nick-repair, and purify the ligated DNA".

Method 2: Fragment gDNA with the Bioruptor® Sonication System

This section describes conditions for shearing genomic DNA with the Bioruptor® UCD-200 *or* the Bioruptor® NGS UCD-600 Sonication System (equipped with an adapter for 0.65-mL tubes) to generate DNA fragments suitable for preparing 100–400-base-read libraries including the 150- and 200-base-read libraries for the Ion Proton™ System. The sonicated DNA is ready for end-repair. Prepare the libraries by adjusting the downstream size-selection of the library molecules.

Sonicate in a refrigerated cold room or on a lab bench. If you are sonicating the DNA on the lab bench, we suggest operating the Bioruptor® Sonication System in a soundproof box to reduce high-frequency noise.

Note: You can use other fragmentation methods such as the Covaris® System. If necessary, reduce the volume of the fragmented DNA by purification with Agencourt® AMPure® XP Reagent at a bead-to-sample volume ratio of 1.8, for volume compatibility with the end-repair reaction.

Genomic DNA

Prepare high-quality, RNA-free genomic DNA (gDNA) using any of a number of commercially available kits. Prepare 100 ng or 1 µg gDNA per library.

Note: For the Ion Proton™ System, use a minimum of 1 µg of DNA for unamplified libraries.

Prepare the samples

Materials and equipment needed

- Bioruptor® Standard Sonication System with accessories (for 12 × 0.5-mL tubes)
- Bioruptor® NGS Sonication System with accessories (for 12 × 0.5-mL tubes)
- Bioruptor® Microtube Attachment and Gearplate (0.5-mL)
- Low TE (10 mM Tris pH 8.0, 0.1 mM EDTA)

1. In a 0.65-mL microcentrifuge tube for the Bioruptor® Sonication System, prepare 100 ng or 1 µg of your genomic DNA preparation in 50 µL of Low TE (pH 8). Close the cap with care so as not to damage the lid and to ensure that the lid forms a tight seal with the tube. Keep the samples on ice.

   IMPORTANT! The material and shape of the tube used for fragmentation of the DNA may have a profound effect on the fragmentation efficiency. This procedure is optimized for 0.65-mL tubes as specified in "Required materials and equipment" on page 12.

2. (*Optional*) In a separate tube, prepare a control sample of 100 ng or 1 µg of control DNA in 50 µL Low TE. Keep the sample on ice.

3. Process ≤12 samples at one time with the 12 × 0.65-mL Bioruptor® Sonication System rotor. If there are <12 samples, load tubes with 50 µL of Low TE to fill all empty slots.

4. Unscrew the removable metal ring from the rotor, insert the 12 tubes, and replace the metal ring finger tight. Do not over-tighten the metal ring.

5. Proceed to "Option 1: Sonicate the DNA with the Bioruptor® CD-200 TS Sonication System" on page 25 in the following section or "Option 2: Sonicate the DNA with the Bioruptor® UCD-600 NGS Sonication System" on page 27.

Option 1: Sonicate the DNA with the Bioruptor® CD-200 TS Sonication System

For 100–300-base-read libraries (including the 150- and 200-base-read libraries for the Ion Proton™ System

1. Set the sonication parameters on the Bioruptor® UCD-200 TS Sonication System. Follow the manufacturer's instructions.

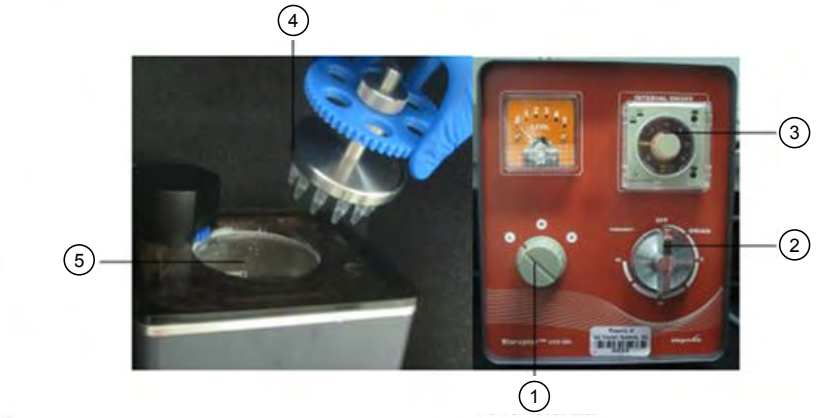
Time ON/OFF	• ON (sonication time, red dial): 0.5 minutes • OFF (cool-down time, green dial): 0.5 minutes
Power Level	L (low)

2. Fill the Bioruptor® Sonication System UCD-200 to 1 cm below the Fill Line with cold (<10°C) water. Add an even 1-cm layer (250 mL) of crushed ice, ensuring that the water is just at the fill line.

3. Set the timer to 15 minutes and sonicate according to the following table:

Sequencing System	Library Size	Number of 15-min Cycles	Total Sonication Time
Ion PGM™ System	300-base-read	1	15 minutes
	200-base-read	3	45 minutes
	100-base-read	5	75 minutes
Ion Proton™ System	200-base-read	3	45 minutes
	150-base-read	5	75 minutes

Between each cycle, remove 1 cm (150 mL) of the water from the Bioruptor® tank and add 250 mL of crushed ice to the Fill Line.



1. Power level dial
2. Timer (15 minutes maximum)
3. INTERVAL [Instrument sonication time (redial); cool down (green dial)]
4. Rotor with twelve 0.65-mL tubes
5. Water bath

4. Remove the tubes from the rotor and store on ice.

5. Proceed to "Assess the fragmentation profile" on page 28.

For 400-base-read libraries

1. Set the sonication parameters on the Bioruptor® UCD-200 TS Sonication System. Follow the manufacturer's instructions.

Time ON/OFF	• ON (sonication time, red dial): 0.5 minutes • OFF (cool-down time, green dial): 1.5 minutes
Power Level	L (Low)

2. Fill the Bioruptor® Sonication System UCD-200 to 1 cm below the Fill Line with cold (<10°C) water. Add an even 1-cm layer (250 mL) of crushed ice, ensuring that the water is just at the fill line.

3. Set the timer to 15 minutes and sonicate.

4. Remove 1 cm (150 mL) of the water from the Bioruptor® tank and add 250 mL of crushed ice to the Fill Line.

5. Set the timer to 9 minutes and sonicate.

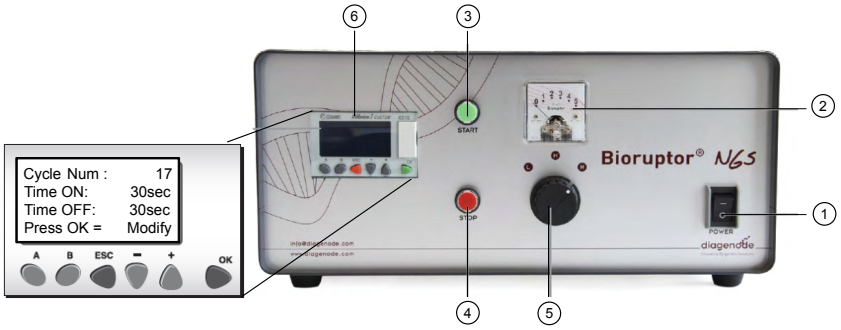
6. Proceed to "Assess the fragmentation profile" on page 28.

Option 2: Sonicate the DNA with the Bioruptor® UCD-600 NGS Sonication System

1. Set the sonication parameters on the Bioruptor® UCD-600 NGS Sonication System. Refer to the instrument manual for detailed instructions.
   a. Press **+** or **-** to select the desired parameter, and press **OK**.
   b. Press **+** or **-** to change the value, and press **OK**.

For 100–200-base-read libraries (including the 150- and 200-base-read libraries for the Ion Proton™ System)

Time ON/OFF	• ON (sonication time): 0.5 minutes • OFF (cool-down time): 0.5 minutes
Cycle Number	17
Power Level	H (High)



For 300- and 400-base-read libraries

Time ON/OFF	• ON (sonication time): 0.5 minutes • OFF (cool-down time): 1.5 minutes
Cycle Number	10[1] (300-base-read) and 9[1] (400-base-read)
Power Level	H (High)

[1] A short centrifugation step after half of the cycle numbers may improve the results.

2. Fill the Bioruptor® Sonication System to just above the Fill Line with water.

3. Switch on the Bioruptor® water cooler and set the temperature to **4°C**.

4. After the set temperature reaches 4°C, insert the rotor containing tubes into the sonicator and press **Start**. Bioruptor® Running will display on the screen. The

total sonication time is 17 minutes for 100–200-base-read libraries, 20 minutes for 300-base-read libraries, and 18 minutes for 400-base-read libraries.

Note: Ensure the temperature of the cooler stays below 10°C during the run.

5. Remove the tubes from the rotor and store on ice.

6. Proceed to "Assess the fragmentation profile" on page 28.

Assess the fragmentation profile

1. Analyze an aliquot of the fragmented DNA as described below to confirm a fragment size range between 50–500 bp, with a peak around 200 bp for 100–200-base-read libraries, and around 300 bp for 300-base-read libraries, and around 400 bp for 400-base-read libraries. See Figures 3–5 in Appendix D, "Bioanalyzer instrument analysis" for example traces.

Input	Bioanalyzer® Instrument Agilent® High Sensitivity DNA Kit	Agarose Gel
100 ng	1 µL	—
1 µg	1 µL of a 1:10 dilution	5 µL

2. Proceed immediately to "End-repair and purify DNA" on page 28.

End-repair and purify DNA

Materials provided in the Ion Plus Fragment Kit

- 5X End Repair Buffer
- End Repair Enzyme

Other materials and equipment

- Nuclease-free Water
- 1.5-mL Eppendorf LoBind® Tubes
- Agencourt® AMPure® XP Kit
- Magnetic rack

End-repair

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

1. Add Nuclease-free Water to the fragmented DNA to bring the **total volume** to the following:

100 ng Input	1 µg Input
79 µL	158 µL

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

Component	Volume by Input	
	100 ng	**1 µg**
Fragmented gDNA (step 1)	79 µL	158 µL
5X End Repair Buffer	20 µL	40 µL
End Repair Enzyme	1 µL	2 µL
Total	**100 µL**	**200 µL**

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

Purify with the Agencourt® AMPure® XP Kit

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 the indicated volume of Agencourt® AMPure® XP Reagent beads (1.8X sample volume) to the sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate at room temperature 5 minutes.

100 ng Input	1 µg Input
180 µL	360 µL

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. 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 magnet, air-dry the beads at room temperature for ≤5 minutes.

7. Remove the tube from the magnet, and add 25 µL of Low TE to the sample. Pipet the mixture up and down 5 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. 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.

9. Proceed to Chapter 4, "Ligate adapters, nick-repair, and purify the ligated DNA".

Ligate adapters, nick-repair, and purify the ligated DNA

Materials provided in the Ion Plus Fragment Library Kit

- 10X Ligase Buffer
- Adapters (for non-barcoded libraries)
- DNA Ligase
- Nick Repair Polymerase
- dNTP Mix
- Low TE

Materials provided in the Ion Xpress™ Barcode Adaptors Kits (for barcoded libraries)

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

Other materials and equipment

- 0.2-mL PCR tubes
- Thermal cycler
- Nuclease-free Water
- 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.

Reaction Setup for Non-barcoded Libraries		
Component	**Volume by Input gDNA**	
	50-100 ng	**1 µg**
DNA	~25 µL	~25 µL
10X Ligase Buffer	10 µL	10 µL
Adapters	2 µL	10 µL
dNTP Mix	2 µL	2 µL
Nuclease-free Water	51 µL	41 µL
DNA Ligase	2 µL	4 µL
Nick Repair Polymerase	8 µL	8 µL
Total	**100 µL**	**100 µL**

Note: Add **both** Ion P1 Adapter and the desired Ion Xpress™ Barcode X adapter to the ligation reaction for barcoded libraries.

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

Reaction Setup for Barcoded Libraries		
Component	**Volume by Input gDNA**	
	50-100 ng[1]	**1 µg[2]**
DNA	~25 µL	~25 µL
10X Ligase Buffer	10 µL	10 µL
Ion P1 Adapter	2 µL	10 µL
Ion Xpress™ Barcode X[3]	2 µL	10 µL
dNTP Mix	2 µL	2 µL
Nuclease-free Water	49 µL	31 µL
DNA Ligase	2 µL	4 µL
Nick Repair Polymerase	8 µL	8 µL
Total	**100 µL**	**100 µL**

[1] All input gDNA 50-100 ng; see above.
[2] All input gDNA 1 µg; see above.
[3] X = barcode chosen.

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	Hold[1]

[1] Not a stopping point; continue directly to the next steps.

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 5 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 ≤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. Mix thoroughly by pipetting the suspension up and down 5 times, then vortex the sample for 10 seconds.

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 Chapter 5, "Size-select the unamplified library".

5 Size-select the unamplified library

This section describes two options for size-selection:

- "Option 1: Size-select the library with the E-Gel® SizeSelect™ Agarose Gel" on page 35
- "Option 2: Size-select the library with the Pippin Prep™ instrument" on page 41

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	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 **http://ioncommunity.lifetechnologies.com** for other library size-selection methods.

Option 1: Size-select the library with the E-Gel® SizeSelect™ Agarose Gel

Start with unamplified library, non-barcoded or barcoded, prepared and purified as described in Chapter 4, "Ligate adapters, nick-repair, and purify the ligated DNA".

Materials and equipment

Materials provided in the Ion Plus Fragment Library 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 the E-Gel® SizeSelect™ Agarose Gel and iBase™ unit

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 the web catalogue page at **www.lifetechnologies.com**.

1. 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.
2. 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.
3. Verify that the iBase™ has the "SizeSelect™ 2%" program. If not, refer to "Downloading upgrade" from the *E-Gel® Technical Guide*.
4. Remove the gel from the package and gently remove the combs from the SizeSelect™ cassette.
5. Insert the gel cassette into the E-Gel® iBase™ unit right edge first.
6. 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.

Load the gel

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-, 400-base-read libraries, 100-bp DNA Ladder for 300-base-read libraries).
- For size-selection of both 1-µg input and 50–100-ng input samples, we recommend running the 1-µg input sample on one gel and the 50–100-ng input on a separate gel.
- If you must run both 1-µg and 50–100-ng input samples on the same gel, follow the guidelines for collection described in step 8 as closely as possible. If both 1-µg and 50–100-ng input samples are collected at exactly the same time, the actual size of collected fragment from the 50–100-ng input sample is always smaller than that of 1-µg input sample.

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

IMPORTANT! 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).

IMPORTANT! Do not load different libraries in adjacent wells, to avoid potential cross contamination.

1. **For 1 µg-input samples:** Before loading, add 20 µL of Low TE to the purified ligated DNA to bring the total volume to 40 µL.

2. Add 20 µL of ligated DNA to the loading well (top row). Use one well for 50–100 ng-input samples.

   Note: Use two adjacent wells for 1-µg input samples, on one side of the gel. For example, use well positions 2 and 3, or well positions 6 and 7.

3. 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.

4. Add 25 µL of Nuclease-free Water to all empty wells in the top row.

5. 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.

Run the gel

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

2. Select the 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 in the following table. 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	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	12–14 minutes
	150-base-read	220 bp	11–14.5 minutes

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

4. 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 2 to 4 with very short run time settings.

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

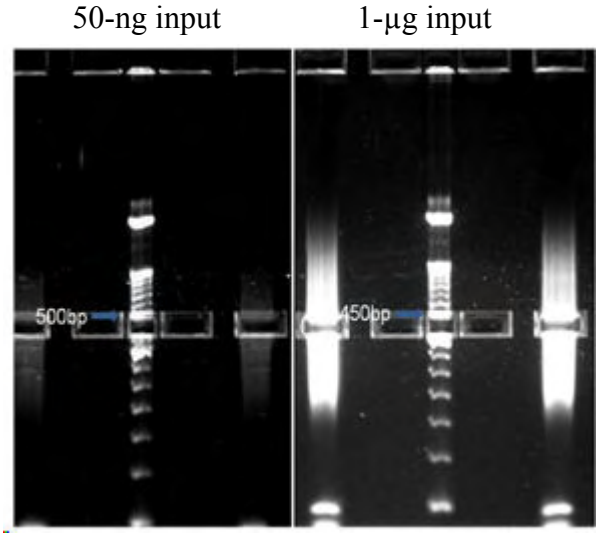
6. Refill the collection wells to 25 µL with ~10 µL of Nuclease-free Water. The water in the wells should form a concave surface. **Do not overfill.**

7. Repeat steps 2-4 with the run time set to 0.5–2.5 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)*.

8. 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):

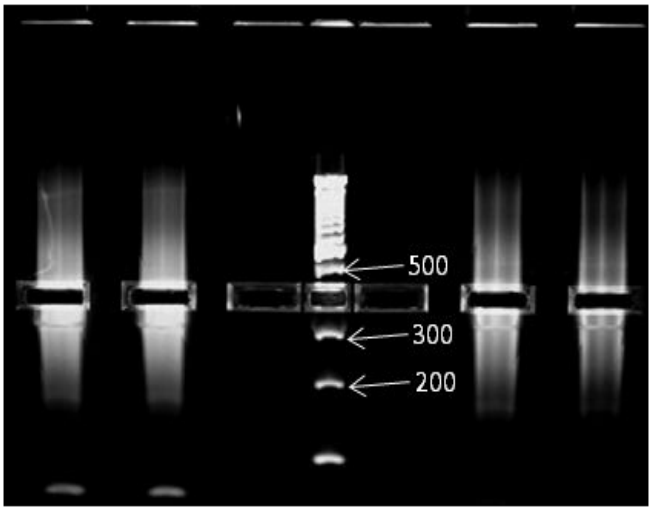
- **For 1 µg-input samples**, stop the run when the 450-bp band migrates into the collection well.
- **For 50–100 ng-input samples**, stop the run when the 500-bp ladder band is at the top edge of the collection well.



400-base-read library gel

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

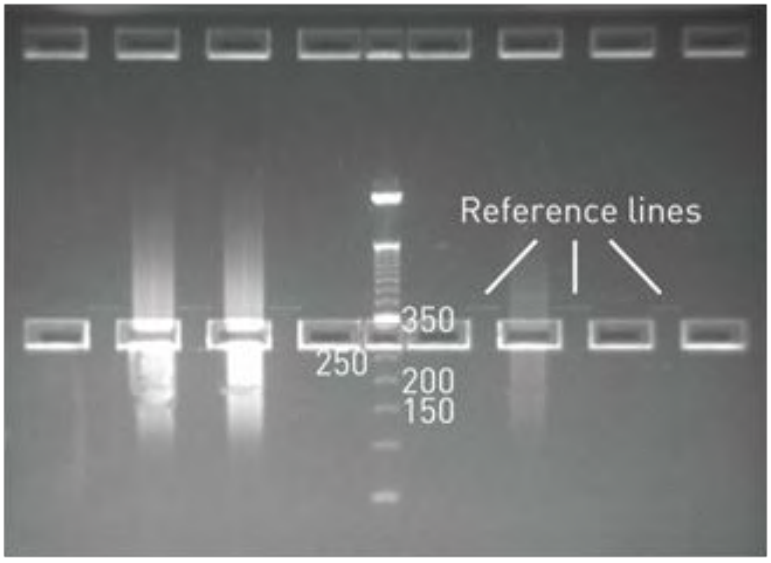
- **For 1 µg-input samples**, stop the run right before the 400-bp band is about to touch the top edge of the collection well.
- **For 50–100 ng-input samples**, stop the run when the 400-bp ladder band is in the middle of the collection well (or 500-bp ladder band is aligned with the reference lines).



300-base-read library gel

200-base-read library (330-bp target peak):

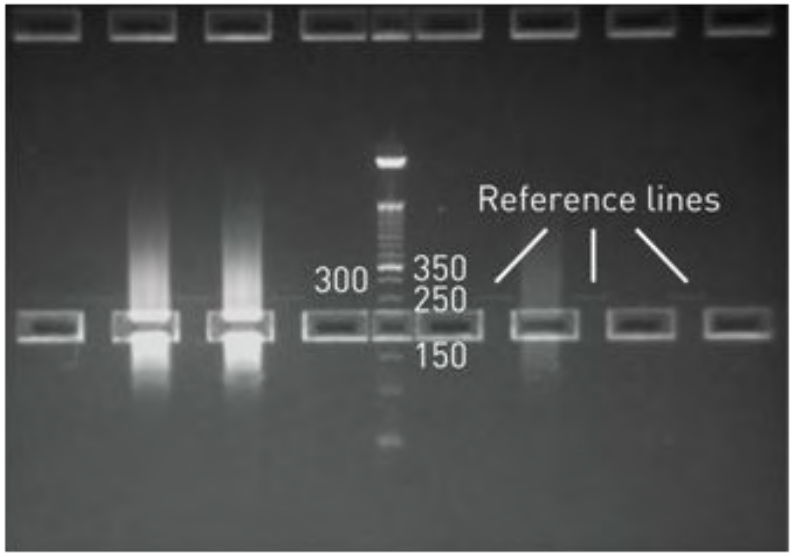
- **For 1 µg-input samples**, stop the run right before the 350-bp band is about to touch the top edge of the collection well.
- **For 50–100 ng-input samples**, stop the run when the 350-bp ladder band has just completely entered the top edge of the collection well.



200-base-read library gel

100–150-base-read libraries (200-220-bp target peak):

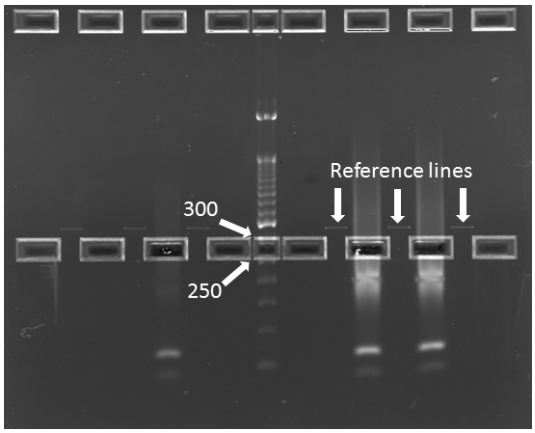
Stop the run when the 200-bp ladder band is in the middle of the collection well.



100-base-read library gel

200-base-read Ion Proton™ libraries (270-bp target peak):

- For 1 µg-input samples, stop the run right before the 300-bp ladder band is about to touch the top edge of the collection well.
- For 50-100 ng-input samples, 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

Collect the sample

1. Collect the solution from the collection wells using a pipette, without piercing the bottom of the well.
2. 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.
3. **For 1 µg-input samples,** combine the recovered DNA from the two appropriate wells. The total volume is ~60 µL.
4. Dispose of the used gels as hazardous waste.
5. If you are using the Ion Library Equalizer™ Kit (for up to 300-base-read libraries only), proceed immediately to Appendix A, "Equalize the library (for up to 300-base-read libraries)". Otherwise, proceed to Chapter 6, "Determine if library amplification is required".

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

Start with unamplified library, non-barcoded or barcoded, prepared and purified as described in Chapter 4, "Ligate adapters, nick-repair, and purify the ligated DNA".

Materials and equipment

Materials provided in the kit

- Low TE

Other materials and equipment

- Pippin Prep™ instrument (Cat. no. 4471271)
- **For 100–300-base-read libraries:** 2% Agarose Gel Cassettes for the Pippin Prep™ instrument; includes Loading Solution, Marker B, and Electrophoresis Buffer (Cat. no. 4472170)
- **For 400-base-read libraries:** 2% Dye Free (DF) Marker L Agarose Gel Cassettes for the Pippin Prep™ instrument; includes Loading Solution/Marker L mix, and Electrophoresis Buffer (Sage Science Cat. no. CDF2010)
- Nuclease-free Water
- Agencourt® AMPure® XP Kit
- Freshly prepared 70% ethanol
- Magnetic rack

IMPORTANT! The protocol below closely follows the Pippin Prep™ instrument manual. Novice users may want to review training videos at **www.sagescience.com/support/** before using the instrument for the first time. Software version 5.8 or higher versions are required to run 2% gels with Marker L.

Define the 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

1. From the cassette type drop-down menu, choose **2% Marker B No Overflow Detection**.
2. 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	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

3. 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.

4. Set the run time for 1.5 hours.

For 400-base-read libraries

1. From the cassette type drop-down menu, choose **2% DF Marker L**.

2. Select the **Tight** collection mode for each lane and then define the BP Target setting for each lane as 475.

3. 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.

4. Set the run time for 1.5 hours.

Prepare the 2% Agarose Gel cassette for the Pippin Prep™ instrument

1. Unwrap the 2% Agarose Gel cassette, and tip the cassette toward the loading wells end to dislodge any air bubbles present around the elution wells. Insert the cassette into the instrument.

2. Remove the two adhesive strips covering the loading wells and wells.

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

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

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

6. Following the instructions in the Pippin Prep™ instrument user guide, confirm that the current across both the separation ports and the elution ports is within specifications.

Load the sample

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

For 100–300-base-read libraries

1. Add 10 µL of Low TE to the purified ligated DNA (20 µL) to bring the volume to 30 µL.

2. Add 10 µL of Loading Solution. The total volume is 40 µL for each sample.

3. Go to the Main screen, then choose the newly generated separation file (or a previously saved file) from the Protocol Name pull-down menu.

4. Remove 40 µL of Electrophoresis Buffer from the loading well of the designated Ref Lane, then load 40 µL of 2% DNA Marker B.

5. 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.

For 400-base-read libraries

1. Add 10 µL of Low TE to the purified ligated DNA (20 µL) to bring the volume to 30 µL.
2. Add 10 µL of Loading Solution/marker mix (labeled Marker L). The total volume is 40 µL for each sample.
3. Go to the Main screen, then choose the newly generated separation file (or a previously saved file) from the Protocol Name pull-down menu.
4. 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.

Run the instrument

1. When the ladder and all samples are loaded, close the lid of the Pippin Prep™ instrument.
2. On the Main screen, press **Start** to initiate the run.
3. 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
4. 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.

Note: If you are using the Ion Library Equalizer™ Kit for library normalization, begin warming the reagents in the kit to room temperature before proceeding.

1. Add 90 µL Agencourt® AMPure® XP reagent (1.5X sample volume) to the sample. Pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, pulse-spin the tube, and incubate the mixture for 5 minutes at room temperature.

   Note: Pulse-spin and place the tube in a magnetic rack for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.

2. Leaving the tube on the magnet, add 500 µL of freshly prepared 70% ethanol to the sample. 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.
3. Repeat step 2 for a second wash.

4. 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.

5. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.

   Note: Assess the dryness of the bead pellet by rotating the plate 90 degrees in the magnet. The pellet should migrate slowly. Do not overdry.

6. If you are using the Ion Library Equalizer™ Kit (for up to 300-base-read libraries only), proceed immediately to "Amplify the library" on page 59. Otherwise, proceed to step 7.

7. Remove the tube from the magnetic rack, and add the indicated volume of Low TE directly to the pellet to disperse the beads.
   - 1 µg input: 50 µL
   - 50–100 ng input: 25 µL

8. Pipet the mixture up and down 5 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.**

10. Proceed to Chapter 6, "Determine if library amplification is required".

6 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: In general, 1 µg-input libraries do not require amplification while libraries from <100 ng inputs do require amplification. However, it can be useful to follow the procedure described in this section for libraries prepared from all input amounts, especially when preparing libraries from a new sample type for the first few times. For the Ion Proton™ System, we recommend amplification for all input amounts, but unamplified 1 µg-input libraries remain an option.

Quantify the unamplified library by qPCR with the Ion Library Quantitation Kit (Cat. no. 4468802). This kit directly determines library concentration so that the library may be appropriately diluted for template preparation.

Determine if amplification is required

1. Determine the concentration of the unamplified library with the Ion Library Quantitation Kit. Follow the instructions in the *Ion Library Quantitation Kit User Guide* (Pub. no. 4468986), and dilute the **unamplified** library for the qPCR as follows.
   - 100 ng-input: 1:1000 dilution
   - 1 µg-input: 1:2000 dilution

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	Chapter 7, "Amplify and purify the library"
No	• Chapter 8, "Qualify non-barcoded libraries" • Chapter 9, "Qualify and pool barcoded libraries"

7 Amplify and purify the library

Materials provided in the Ion Plus Fragment Library Kit

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

Other materials and equipment

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

Amplify the library

1. Adjust the volume of the unamplified library as described below.

Size-selection Method	E-Gel® SizeSelect™ Agarose Gel		Pippin Prep™ Instrument	
Library input amount	**50–100 ng**	**1 µg**	**50–100 ng**	**1 µg**
Volume to amplification rxn (step 2)	25 µL	50 µL	25 µL	50 µL

2. Combine the following reagents in an appropriately sized tube and mix by pipetting up and down.

Component	Volume by Input DNA	
	50-100 ng	1 µg
Platinum® PCR SuperMix High Fidelity	100 µL	200 µL
Library Amplification Primer Mix	5 µL	10 µL
Unamplified library	25 µL	50 µL
Total	**130 µL**	**260 µL**

3. Split the 130-µL or 260-µL reaction mix equally into multiple 0.2-mL PCR tubes to adjust for the maximum reaction volume recommended by the manufacturer of your thermal cycler.

   For example, the recommended maximum PCR volume for the Veriti® 96-well Thermal Cycler and the Dual 96-well GeneAmp® PCR System 9700 is 80 µL and 100 µL, respectively. Therefore, the 130-µL reaction can be split into two PCR tubes, each containing 65 µL, and the 260-µL reaction can be split into either three tubes containing 86 µL or four tubes containing 65 µL.

4. Place the tubes into a thermal cycler and run the following PCR cycling program. Set the number of cycles according to the second table.

   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
Holding	Denature	95°C	5 min
Cycling[1]	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	70°C	1 min
Holding	—	4°C	Hold[2]

[1] Set the number of cycles according to the following table.

[2] Not a stopping point; continue directly to the next steps.

Number of Cycles by Library Input	
50-100 ng	1 µg
8	5

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

Purify the library

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 each sample:

Volume of Agencourt® AMPure® XP Reagent			
Input	**100–150-base-read library (1.5X Sample volume)**	**200–300-base-read library (1.2X Sample volume)**	**400-base-read library (1X Sample volume)**
50–100 ng	195 µL	156 µL	130 µL
1 µg	390 µL	312 µL	260 µL

2. Pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate the mixture for 5 minutes at room temperature.

3. 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.

4. 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.

5. Repeat step 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, and add 20 µL of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down 5 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 Chapter 8, "Qualify non-barcoded libraries".
For barcoded libraries, proceed to Chapter 9, "Qualify and pool barcoded libraries".

8 Qualify non-barcoded libraries

For barcoded libraries, go to Chapter 9, "Qualify and pool barcoded libraries".

Assess the size distribution of the library

Analyze an aliquot of the library on the Bioanalyzer® instrument with an Agilent® High Sensitivity DNA Kit, as indicated in the following table.

See Figures 6–13 in Appendix D, "Bioanalyzer® instrument analysis" for example traces.

Library Type	Unamplified		Amplified	
Input amount	**100 ng**	**1 µg**	**50–100 ng**	**1 µg**
Library aliquot	1 µL	1 µL, 1:5	1 µL, 1:10	1 µL, 1:10

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

Quantify and dilute the library

Quantify the library and dilute the library to a 100 pM standard concentration suitable for template preparation.

Unamplified libraries: Determine the library dilution by qPCR with the Ion Library Quantitation Kit (Cat. no. 4468802).

Amplified libraries: Determine the library dilution by Bioanalyzer® instrument analysis *or* by qPCR.

Quantitation Method	Features
Ion Library 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 Quantitation Kit is recommended for unamplified or low-yield libraries. Libraries with insufficient material for detection by Bioanalyzer® instrument analysis 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 Quantitation Kit accurately quantifies the properly adapted libraries with minimal impact from background material.
Bioanalyzer®instrument instrument analysis	• Determination of a molar concentration for the library, from which the library dilution is calculated. • Concentration is part of the output of the Bioanalyzer® instrument analysis to assess the library size distribution, 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 Quantitation Kit to determine the library dilution.
- Use Bioanalyzer® instrument analysis to assess the size distribution of the library.

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

1. Use the Ion Library Quantitation Kit (Cat. no. 4468802) to determine the library concentration in pM by quantitative real-time PCR (qPCR). Follow the instructions in the *Ion Library Quantitation Kit User Guide* (Pub. no. 4468986).

2. Determine the dilution factor that results in a concentration of ~100 pM. This concentration is suitable for downstream template preparation.
   Use 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 mixed with 149 µL of Low TE (1:150 dilution) yields approximately 100 pM. Use this library dilution for template preparation.

   Note: If your non-diluted library has a concentration lower than 100 pM, determine whether amplification is necessary to provide sufficient yield for your experimental needs. Refer to Chapter 6, "Determine if library amplification is required".

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

Determine the library concentration from Bioanalyzer® instrument analysis (amplified libraries only)

1. From the Bioanalyzer® instrument analysis used to assess the library size distribution, determine the molar library concentration in pmol/L 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.
2. Determine the dilution factor that results in a concentration of ~100 pM. This concentration is suitable for downstream template preparation.
   Use 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 mixed with 149 µL of Low TE (1:150 dilution) yields approximately 100 pM. 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. Template preparation documentation is available on the Ion Community at **http://ioncommunity.lifetechnologies.com**. Follow the links under **Protocols ▸ Prepare Template ▸ Prepare Template User Guides and Quick Reference**.

9 Qualify and pool barcoded libraries

Pooling barcoded libraries in equimolar amounts ensures equal representation of each barcoded library in the sequencing run. Barcoded libraries are individually quantitated and pooled. This section describes alternative pooling procedures according to the library quantitation method.

Note: Unamplified libraries must be quantitated for pooling with the Ion Library Quantitation Kit (Cat. no. 4468802).

For non-barcoded libraries, follow Chapter 8, "Qualify non-barcoded libraries".

Assess the size distribution of individual barcoded libraries

Analyze an aliquot of each barcoded library with an Agilent® High Sensitivity DNA Kit, as indicated in the following table. Follow the manufacturer's instructions.

See Figures 6–13 in Appendix D, "Bioanalyzer instrument analysis" for example traces.

Library Type	Unamplified		Amplified	
Input amount	**100 ng**	**1 µg**	**50–100 ng**	**1 µg**
Library aliquot	1 µL	1 µL, 1:5	1 µL, 1:10	1 µL, 1:10

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

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

1. Use the Ion Library Quantitation Kit (Cat. no. 4468802) to determine the library concentration by quantitative real-time PCR (qPCR) for each individual barcoded library. Follow the instructions in the *Ion Library Quantitation Kit User Guide* (Cat. no. 4468986).

2. Determine the dilution factor that gives a concentration of ~100 pM. This concentration is suitable for downstream template preparation.
   Use 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 mixed with 149 µL of Low TE (1:150 dilution) yields approximately 100 pM. Use this library dilution for template preparation.

3. Dilute each barcoded library to 100 pM.

   Note: If a non-diluted barcoded library has a concentration lower than 100 pM, determine whether amplification is necessary to provide sufficient yield for your experimental needs. Refer to Chapter 6, "Determine if library amplification is required". Otherwise dilute barcoded libraries to the highest possible equimolar concentration.

4. 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.

Pool barcoded libraries using Bioanalyzer® instrument quantitation (amplified libraries only)

1. From the Bioanalyzer® instrument analysis used to assess the individual barcoded library size distribution, determine the molar concentration in pmol/L of each barcoded library 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.

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, then 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. Determine the dilution factor that gives a concentration of ~100 pM. This concentration is suitable for downstream template preparation.
   Use 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 mixed with 149 µL of Low TE (1:150 dilution) yields approximately 100 pM. 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. Template preparation documentation is available on the Ion Community at **http://ioncommunity.lifetechnologies.com**. Follow the links under **Protocols ▸ Prepare Template ▸ Prepare Template User Guides and Quick Reference**.

Equalize the library (for up to 300-base-read libraries)

Note: The Ion Library Equalizer™ Kit (Cat. no. 4482298) provides a method for normalizing library concentration at ~100 pM without the need for quantification, for up to 300-base-read libraries only.

E-Gel® size-selected libraries require purification before equalization (described below). To equalize the library, amplify then capture the library on Equalizer™ Beads. After elution of the equalized library, proceed directly to combining libraries or template preparation.

Materials and Equipment

Materials provided in the Ion Plus Fragment Library Kit

- Platinum® PCR SuperMix High Fidelity

Materials provided in the Ion Library Equalizer™ Kit

- Equalizer™ Primers
- Equalizer™ Capture Solution
- Equalizer™ Elution Buffer
- Equalizer™ Beads
- Equalizer™ Wash Buffer

Other materials and equipment

- E-Gel® agarose gel size selection only: Agencourt® AMPure® XP Kit
- 0.2-mL PCR tubes
- Thermal cycler
- Magnetic rack

E-Gel® agarose gel size selection only: Purify the library

If you performed E-Gel® agarose gel size selection (see "Option 1: Size-select the library with the E-Gel® SizeSelect™ Agarose Gel" on page 35), purify the library before equalization as described below. If you performed size selection with the Pippin Prep™ instrument, proceed to "Amplify the library" on page 59.

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

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1. Before starting, begin warming the reagents in the Ion Library Equalizer™ Kit to room temperature.

2. Depending on the volume of DNA from E-Gel® agarose gel size selection, add the following volume of Agencourt® AMPure® XP Reagent:

Volume of size-selected DNA	Volume of Agencourt® AMPure® XP Reagent (1.8X sample volume)
~30 µL	54 µL
~60 µL	108 µL

3. Pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, pulse-spin the tube, and incubate the mixture for 5 minutes at room temperature.

4. 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.

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

6. Repeat step 5 for a second wash.

7. 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.

8. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.

   Note: Assess the dryness of the bead pellet by rotating the plate 90 degrees in the magnet. The pellet should migrate slowly. Do not overdry.

Amplify the library

1. Warm the reagents in the Ion Library Equalizer™ Kit to room temperature before use.
2. Remove the tube from the magnet, and add 50 µL of Platinum® PCR SuperMix High Fidelity (black cap) and 2 µL of Equalizer™ Primers to the bead pellet. The SuperMix and primers may be combined before addition. Pipet the mixture up and down 5 times to mix thoroughly.

   IMPORTANT! Do **not** use the Library Amplification Primer Mix provided in the Ion Plus Fragment Library Kit.
3. Place the tube back on the magnet for at least 2 minutes, then carefully transfer ~50 µL of supernatant to a 0.2-mL PCR tube, without disturbing the pellet.
4. Seal the tube, place it in the thermal cycler, and run the following program.

   Note: During cycling, proceed to preparing the beads.

Stage	Step	Temperature	Time
Holding	Denature	95°C	5 min
Cycling[1]	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	70°C	1 min
Holding	—	4°C	Hold[2]

[1] Set the number of cycles according to the following table.

[2] Not a stopping point; continue directly to the next steps.

Number of cycles by library input	
50–100 ng	**1 µg**
8	5

Prepare the Equalizer™ Beads

1. Bring the Equalizer™ Beads to room temperature and mix thoroughly.
2. Pipet 3 µL of beads/reaction into a clean tube and add 6 µL/reaction of Equalizer™ Wash Buffer.

   Note: Beads for multiple reactions may be prepared in bulk.
3. Place the tube in a magnetic rack for 3 minutes or until the solution is completely clear.
4. Carefully remove and discard the supernatant without disturbing the pellet.

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

   Note: You can store the beads in Equalizer™ Wash Buffer at 4°C for up to 1 month until use. After 1 month, beads may be rewashed.

Add Equalizer™ Capture Solution to the amplified library

1. After thermal cycling, add 10 µL of Equalizer™ Capture Solution to the amplification reaction.
2. Set the pipette volume to 40 µL and pipet the mixture up and down 5 times to mix thoroughly.
3. Incubate at room temperature for 5 minutes.

Add the Equalizer™ Beads and wash

1. Mix the washed Equalizer™ Beads by gentle vortexing or pipetting up and down.
2. Add 6 µL beads to each plate well containing the capture reaction.
3. Set the pipette volume to 40 µL and pipet the mixture up and down 5 times to mix thoroughly.
4. Incubate at room temperature for 5 minutes.
5. Place the tube in the magnet and incubate for 2 minutes or until the solution is clear.
6. Carefully remove and discard the supernatant without disturbing the pellet, and add 150 µL of Equalizer™ Wash Buffer to the reaction.
7. Turn the tube around twice in the magnet to wash the beads, or remove from magnet and gently pipet up and down 5 times with a pipettor set at 100 µL. Return the tube to the magnet.
8. With the tube still in the magnet, carefully remove and discard the supernatant without disturbing the pellet, and add 150 µL of Equalizer™ Wash Buffer to the reaction.
9. Turn the tube around twice in the magnet to wash the beads, or remove from magnet and gently pipet up and down 5 times with a pipettor set at 100 µL. Return the tube to the magnet.
10. With the tube still in the magnet, carefully remove and discard the supernatant.

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

Elute the equalized library

1. Remove the tube from the magnet and add 100 µL of Equalizer™ Elution Buffer to the pellet. Pipet up and down 5 times to mix thoroughly.
2. Seal the tube and incubate in a thermal cycler at 35°C for 5 minutes.
3. Place the tube in the magnet and incubate at room temperature for 5 minutes or until the solution is clear.
4. The supernatant contains the equalized library. Proceed immediately to template preparation, or combine and/or store the library as described below.

   Note: The final concentration of each equalized library is ~100 pM.

(Optional) Combine equalized libraries

If you are combining equalized libraries, remove the supernatant containing each library from the beads and combine them without dilution. Combined libraries may be stored in a sealed plate at 4–8°C.

Store equalized libraries

Undiluted equalized libraries (combined or uncombined) may be stored in a sealed plate or tube at 4–8°C. If uncombined libraries are stored with beads, perform the following steps each time before taking aliquots:

1. Cover the plate and incubate in a thermal cycler at 35°C for 5 minutes.
2. Place the plate in the magnet and incubate at room temperature for 5 minutes or until the solution is clear.

Template preparation

Follow the recommendations for diluting your library for template preparation in the template user guide appropriate to your Ion PGM™ or Ion PI™ Template OneTouch™ 2 Kit.

Template preparation documentation is available on the Ion Community at **http://ioncommunity.lifetechnologies.com**. Follow the links under **Protocols ▸ Ion PGM™ Sequencer ▸ Prepare Template** or **Protocols ▸ Ion Proton™ Sequencer ▸ Prepare Template**.

B

Fragment DNA with the Covaris® System

Fragment gDNA with the Covaris® M220 sonicator

This section describes shearing of genomic DNA with the Covaris® M220 Focused-ultrasonicator™ system to generate DNA fragments suitable for 100–400-base-read libraries for sequencing on the Ion PGM™ System. The sonicated DNA is ready for end-repair. Prepare the libraries by adjusting the downstream size-selection of the library molecules.

For detailed instructions on using the Covaris® System, including loading and unloading the Covaris® microTUBE™ Tube in the microTUBE™ holder, see the manufacturer's instructions.

Genomic DNA

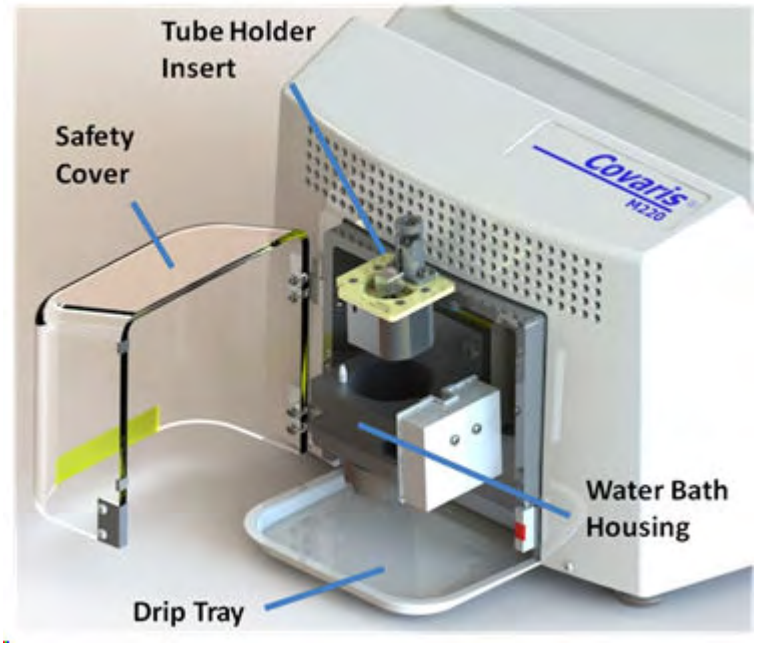
Prepare high-quality, RNA-free genomic DNA (gDNA) using any of a number of commercially available kits. Prepare 100 ng–1 µg gDNA per library.

Materials and equipment needed

- Covaris® M220 Focused-ultrasonicator
- SonoLab 7 Software
- Covaris® Holder microTUBE™ M220
- Covaris® microTUBE™ Screw-Cap Tube
- Covaris® microTUBE™ Prep Station
- Covaris® AFA-grade Water ***or*** Highly Purified Water (≥ASTM Type III or ISO Grade 3).
- Low TE (10 mM Tris pH 8.0, 0.1 mM EDTA)
- Eppendorf LoBind® Tubes (1.5 mL)

Note: Settings for processing 50-µl samples in microTUBE™ Screw-Cap Tubes can be found on the Covaris® website: **http://covarisinc.com/products/afa-ultrasonication/m-series/m220-ion-compatible/**

Procedure



1. Open the Safety Cover and place the microTUBE™ Holder insert into the water bath housing.

2. Fill the Covaris® water bath housing with Covaris® AFA-grade Water using the provided wash bottle. Continue adding water until the water reaches the top surface of the microTUBE™ Holder (~15 mL) and the water level indicator in the SonoLab 7 Software turns to green.

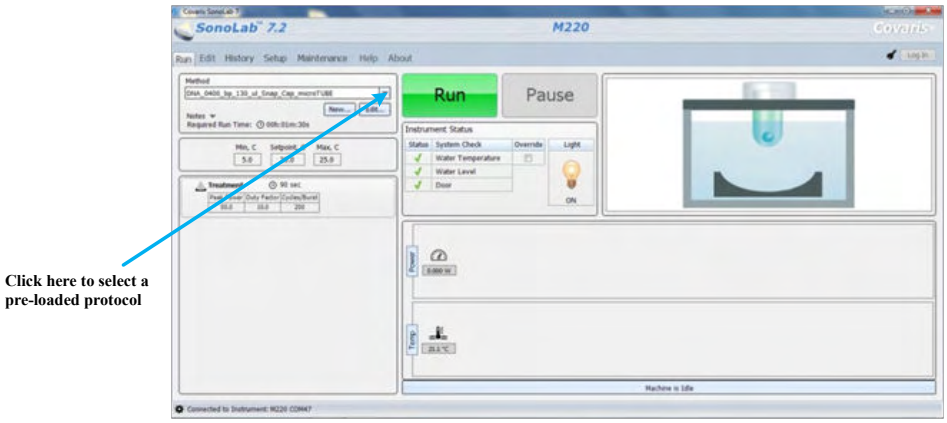
3. Dilute DNA in an Eppendorf LoBind® Tube:

Component	Amount
DNA	100 ng or 1 µg
Low TE	Variable
Total	**50 µL**

4. Place a Covaris® microTUBE™ Screw-Cap Tube into the Prep Station. Unscrew the cap and use a tapered pipette tip to slowly transfer the diluted DNA sample into the microTUBE™ Tube. Be careful not to introduce a bubble into the bottom of the tube. Close the microTUBE™ Tube.

5. Select the preloaded protocol in the SonoLab software corresponding to the desired library size. Temperature is preprogrammed for each protocol and will be automatically regulated by the M220 once the protocol selected:

Library Size	Protocol
400-base-read	Ion_Torrent_400bp_50ul_ScrewCap_microTUBE
300-base-read	Ion_Torrent_300bp_50ul_ScrewCap_microTUBE
200-base-read	Ion_Torrent_200bp_50ul_ScrewCap_microTUBE
100-base-read	Ion_Torrent_100bp_50ul_ScrewCap_microTUBE



6. You may also enter the settings manually, as shown in the following table:

Library Size	Mean Fragment Size	Peak Incident Power (PIP)	Duty Factor	Cycles per Burst	Treatment Time	Temp	Sample Volume
400 bp	410 bp	50 W	20%	200	60 sec	20°C	50 µL
300 bp	320 bp	50 W	20%	200	100 sec	20°C	50 µL
200 bp	260 bp	50 W	20%	200	130 sec	20°C	50 µL
100 bp	150 bp	50 W	20%	200	375 sec	20°C	50 µL

7. Place the Covaris® microTUBE™ in the holder, close the Safety Cover and click on "Run" in the SonoLab software.

8. Once the treatment is finished, place the Covaris® microTUBE™ Tube into the Prep Station. Unscrew the cap, slowly remove the sheared DNA, and transfer it into a new 1.5-mL Eppendorf LoBind® Tube.

Assess the fragmentation profile

1. Analyze an aliquot of the fragmented DNA as described below to confirm a fragment size with a peak around 150 bp for 100-base-read libraries, 260 bp for 200-base-read libraries, and 320 bp for 300-base-read libraries, and 410 bp for 400-base-read libraries.

Input DNA	Bioanalyzer® Instrument Agilent®High Sensitivity DNA Kit	Agarose Gel
100 ng	1 µL	—
1 µg	1 µL of a 1:10 dilution	5 µL

2. Proceed immediately to “End-repair and purify DNA” on page 28.

Fragment gDNA with the Covaris® S2 and S220 sonicators

This section describes sonication of genomic DNA with the Covaris® S2 or S220 Systems to generate DNA fragments suitable for up to 400-base-read libraries for sequencing on the Ion PGM™ System. The sonicated DNA is ready for end-repair. For detailed instructions on using the Covaris® System, including loading and unloading the Covaris® microTUBE™ Tube in the microTUBE™ holder, see the manufacturer's instructions.

Materials and equipment needed

- Covaris® S2 or S220 System (110 V for U.S. customers; 220 V for international customers
- Covaris® microTUBE™ Screw-Cap Tubes
- Low TE (10 mM Tris pH 8.0, 0.1 mM EDTA)
- Ethylene glycol
- Eppendorf LoBind® Tubes (1.5 mL)
- (*Optional*) Shear Buffer
  Provided with the Ion Plus Library Kit for AB® Library Builder™ System

Procedure

IMPORTANT! Set the chiller temperature to between 2°C and 5°C to ensure that the temperature reading in the water bath displays 5°C. The circulated water chiller, not the water bath itself, should be supplemented with 20% ethylene glycol.

1. Fill the Covaris® water bath to level 12, then degas the water bath for 30 minutes before shearing. When you place the tube in the holder, ensure that the base of the cap is at water level and the glass portion of the tube is completely submerged.

2. Dilute DNA in an Eppendorf LoBind® Tube:

Component	Amount
DNA	100 ng or 1 µg
Low TE	Variable
Total	**130 µL**

3. Place a Covaris® microTUBE™ Tube into the loading station. Keep the cap on the tube and use a tapered pipette tip to slowly transfer the diluted DNA sample through the pre-split septa. Be careful not to introduce a bubble into the bottom of the tube.

4. Shear the DNA using the following shearing conditions:

Covaris® S2 Settings

Condition	100–250-bp Fragments	350-bp Fragments	400-bp Fragments
Number of cycles	6	2	2
Bath temperature	5°C	5°C	5°C
Bath temperature limit	12°C	10°C	10°C
Mode	Frequency sweeping	Frequency sweeping	Frequency sweeping
Water quality testing function	Off	Off	Off
Duty cycle	10%	10%	10%
Intensity	5	5	5
Cycles/burst	100	100	100
Treatment Time	60 sec	45 sec	40 sec

Covaris® S220 Settings

Condition	100–250-bp Fragments	350-bp Fragments	400-bp Fragments
Bath temperature	5°C	5°C	5°C
Duty cycle	10%	10%	10%
Peak Incident Power (PIP)	175 W	175 W	175 W
Cycles/burst	100	100	100
Treatment Time	360 sec	90 sec	80 sec

5. Place the Covaris® microTUBE™ Tube into the loading station. Keeping the snap-cap on, insert a pipette tip through the pre-split septa, slowly remove the sheared DNA, and transfer the DNA into a new 1.5-mL Eppendorf LoBind® Tube.

6. Either dilute an aliquot 1:50 in low TE and analyze using a Bioanalyzer® High Sensitivity DNA LabChip® Kit, ***or*** analyze an undiluted aliquot on an agarose gel. Bioanalyzer® instrument peaks should be around 200 bp for 100–250-bp fragments, around 320 bp for 350-bp, and around 410 bp for 400-bp fragments.

7. Transfer each sheared DNA sample to a new individual 1.5-mL Eppendorf LoBind® tube.

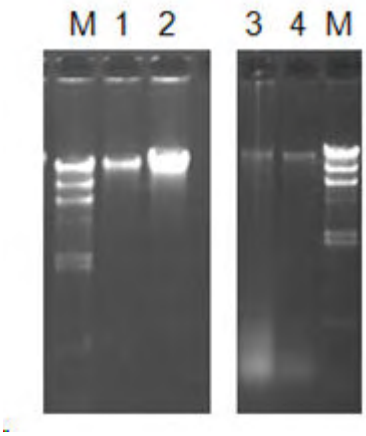
Note: For DNA samples 150 ng or less, bring the sample volume to ~80 µL using Low TE if necessary. If the volume of sheared DNA is >80 µL, concentrate the DNA to ~80 µL in a SpeedVac® concentrator. If a SpeedVac® concentrator is not available, perform an AMPure® XP Reagent purification step (see "Purify the fragmented DNA" on page 23). Use 1.8X sample volume of the AMPure® XP Reagent and elute the DNA in a volume of ~80 µL. Alternatively, use the end-repair reaction conditions for 1 µg input DNA, which accommodate a 158-µL volume of DNA.

8. Proceed immediately to "End-repair and purify DNA" on page 28.

C Evaluate the quality of the genomic DNA

Assess the integrity and size by gel electrophoresis

We recommend checking the integrity and size of your DNA preparation by gel electrophoresis. Use of a spectrophotometer to assess DNA quality can be misleading, because many molecules absorb in ultraviolet light.



Examples of genomic DNA preparations

Examples of high-quality DNA with no contaminating RNA (lanes 1 and 2), compared to lower quality samples containing RNA contamination (lanes 3 and 4). The RNA runs as a diffuse smear at the bottom of the gel. M is a lambda *Hind*III-digest molecular weight marker.

If your DNA preparation shows RNA contamination, treat it with RNase I, as described in the following section.

(Optional) Treat the DNA with RNase I

Treat your purified DNA with RNase I only if RNA contamination is evident.

Required materials and equipment

- RNase I
- PureLink® columns from the PureLink® Genomic DNA Kit, or another purification technology compatible with high molecular-weight DNA

Note: RNase I is recommended. We do not recommend RNase A, which is a site-specific endonuclease and therefore does not degrade the RNA sufficiently to remove it.

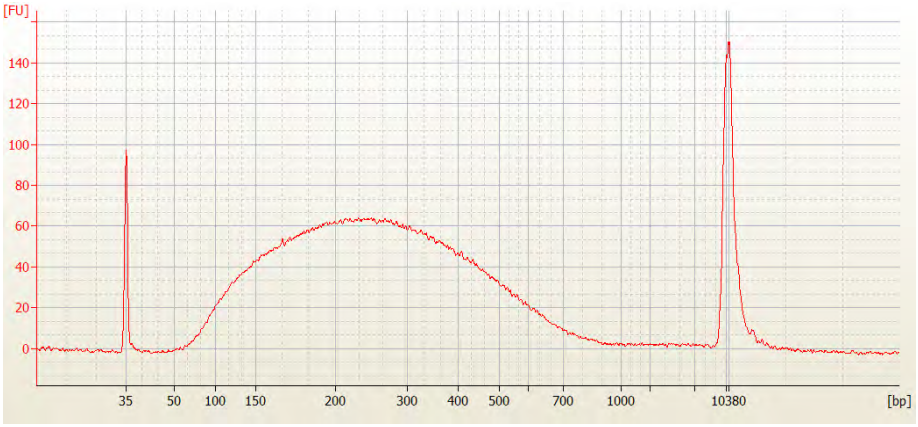
1. Treat the DNA with RNase I according the manufacturer's instructions.
2. Remove the buffer used for RNase I treatment. For example, use a PureLink® spin column from the PureLink® Genomic DNA Kit (follow the "Purification Procedure Using Spin Columns" protocol provided in the *PureLink® Genomic DNA Kits User Guide*).

   IMPORTANT! The buffer used for RNase I treatment interferes with library construction.

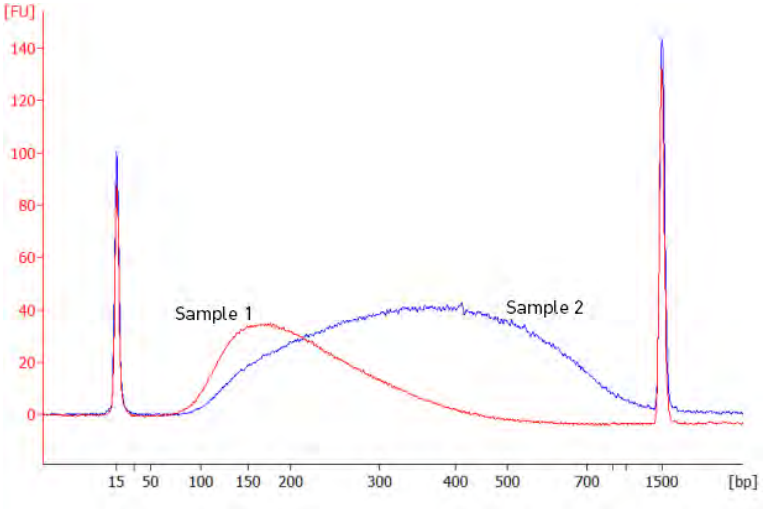
D Bioanalyzer® instrument analysis

DNA fragmented with Ion Shear™ Plus Reagents

Figure 1 Bioanalyzer® instrument analysis of genomic DNA fragmented for 200-base-read libraries



Panel A, Optimal fragmentation profile: 1 µg of *E. coli* DH10B DNA was fragmented with the Ion Shear™ Plus Reagents with a reaction time adjusted for 200-base-read libraries. Analysis was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

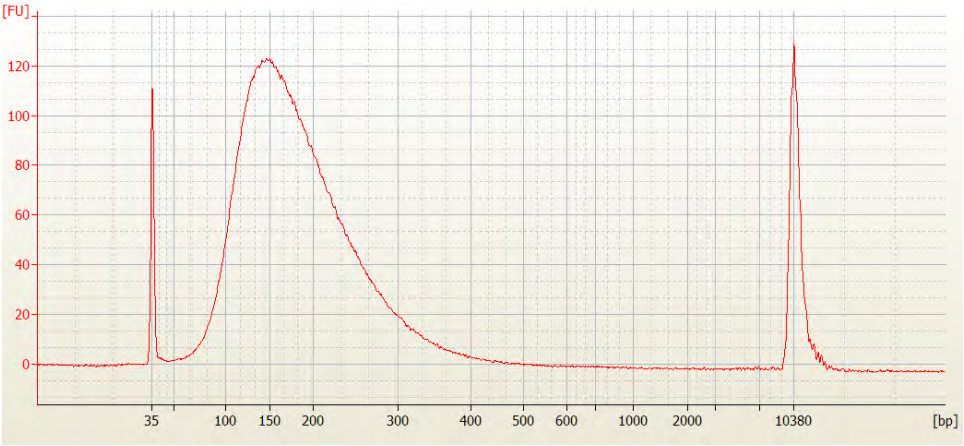


Panel B, Over- and under-digestion profiles: Examples of over-digested (Sample 1, red) and under-digested (Sample 2, blue) gDNA fragments for a 200-base-read

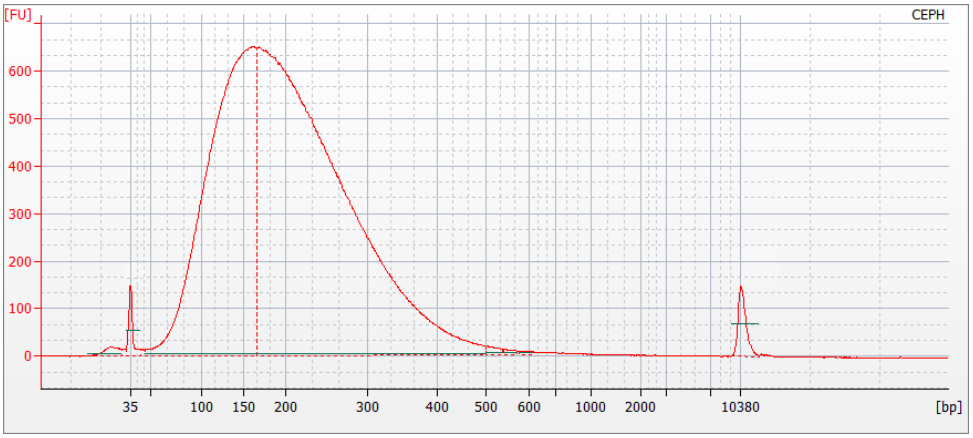
library. Either of these fragmentation profiles can be used for library construction. The expected library yield is greater than that from optimally fragmented DNA using either the Bioruptor® or Covaris® systems, due to the higher ligation efficiency of fragments generated using Ion Shear™ Plus Reagents.

Figure 2 Bioanalyzer® instrument analysis of genomic DNA fragmented for 100-base-read libraries

Genomic DNA was fragmented with the Ion Shear™ Plus Reagents with a reaction time adjusted for 100-base-read libraries. Analysis was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.



Panel A, Fragment profile of 1 µg of *E. coli* DH10B DNA supplied in the Ion Control Materials 200 Kit (Cat. no. 4471249) for Ion PGM™ System sequencing.



Panel B, Fragment profile of 1 µg of human gDNA CEPH Individual 1347-02 supplied in the Ion Proton™ Controls Kit (Cat. no. 4478328) for Ion Proton™ System sequencing.

DNA fragmented with the Bioruptor® System

Figure 3 Bioanalyzer® instrument analysis of genomic DNA fragmented for 400-base-read libraries

1 µg of *E. coli* DH10B DNA was fragmented with the Bioruptor® NGS System for 20 minutes. Analysis was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

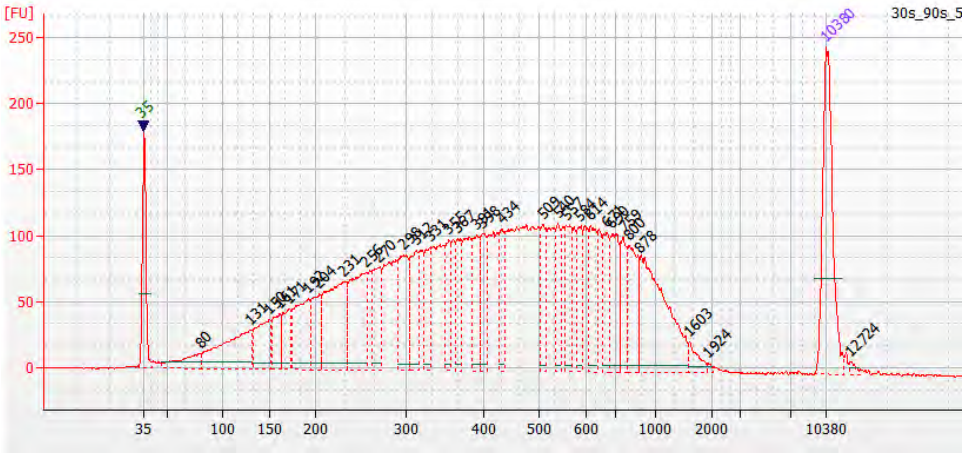


Figure 4 Bioanalyzer® instrument analysis of genomic DNA fragmented for 300--base--read libraries

1 µg of *E. coli* DH10B DNA was fragmented with the Bioruptor® NGS System for 20 minutes. Analysis was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

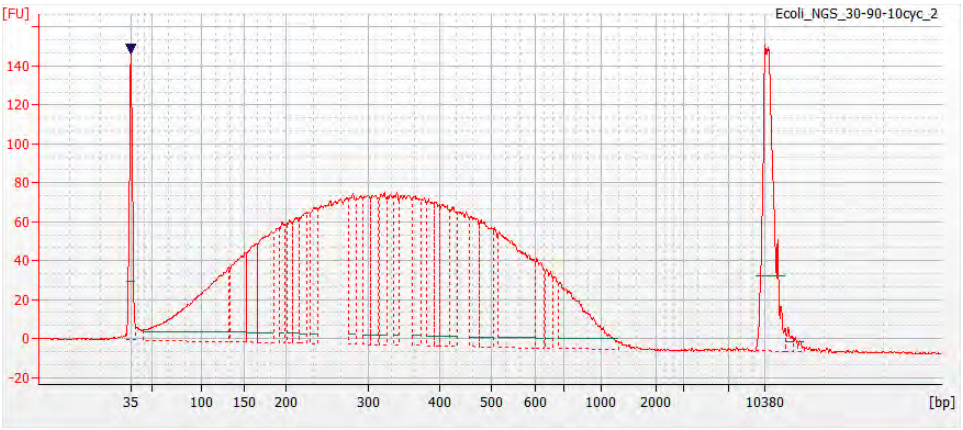
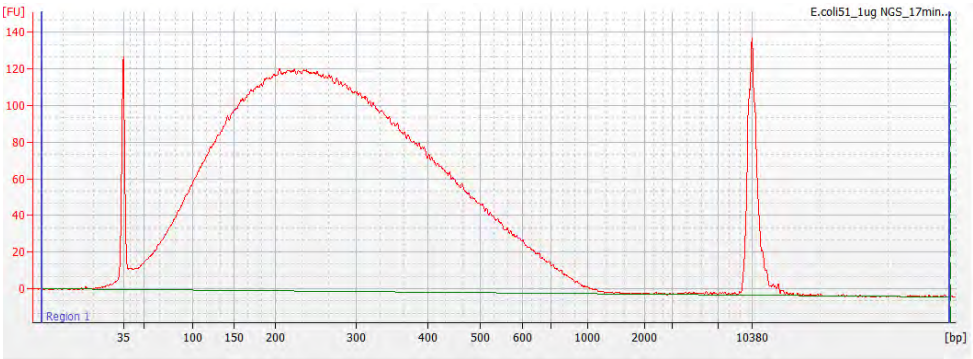


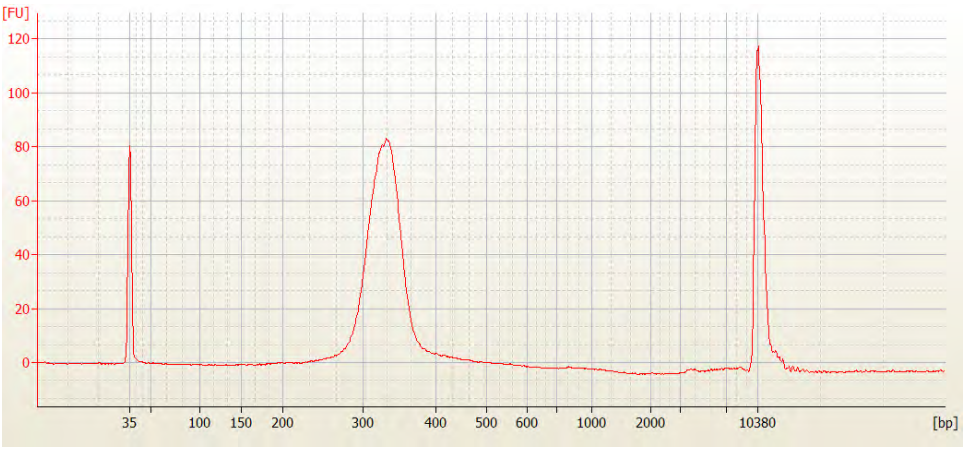
Figure 5 Bioanalyzer® instrument analysis of genomic DNA fragmented for 100- and 200-base-read libraries

1 µg of *E. coli* DH10B DNA was fragmented with the Bioruptor® NGS System for 17 minutes. Analysis was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

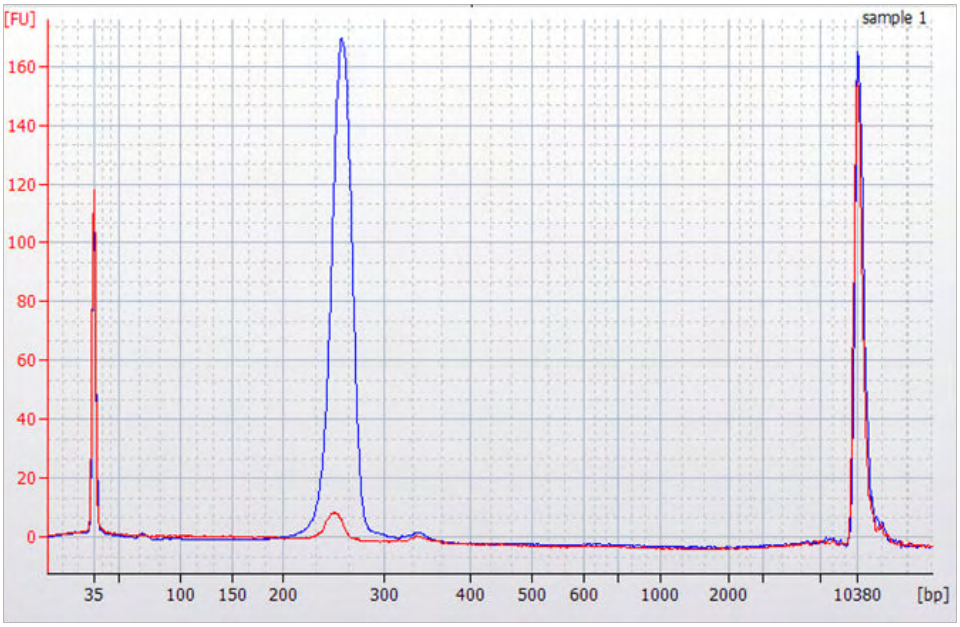


DNA fragmented with Ion Shear™ Plus Reagents and size-selected with E-Gel® SizeSelect™ Agarose Gel

Figure 6 Bioanalyzer® instrument analysis of an unamplified 200-base-read library



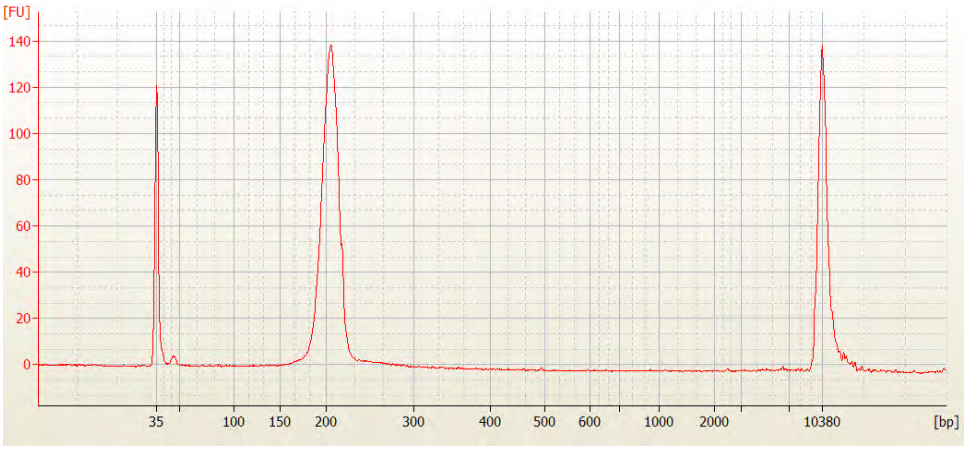
Panel A, 1 µg of *E. coli* DH10B DNA was fragmented with the Ion Shear™ Plus Reagents with a reaction time adjusted for 200-base-read libraries. An unamplified library was prepared from the fragmented DNA and size-selected with the E-Gel® SizeSelect™ 2% Agarose for 200-base-read libraries (330-bp length), as described in this user guide. Analysis was with the Agilent® High Sensitivity DNA Kit.



Panel B, 1 µg (blue), or 50 ng (red), of *E. coli* DH10B DNA was fragmented with Ion Shear™ Plus Reagents with a reaction time adjusted for 200-base-read libraries. An unamplified library was prepared from the fragmented DNA and size-selected with the E-Gel® SizeSelect™ 2% Agarose Gel for 200-base-read libraries (270-bp length), as described in this user guide. Analysis was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

Figure 7 Bioanalyzer® instrument analysis of an unamplified 100-base-read library

1 µg of *E. coli* DH10B DNA was fragmented with the Ion Shear™ Plus Reagents with a reaction time adjusted for 100-base-read libraries. An unamplified library was prepared from the fragmented DNA and size-selected with the E-Gel® SizeSelect™ 2% Agarose for 100-base-read libraries (200-bp length), as described in this user guide. Analysis was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.



DNA fragmented with Ion Shear™ Plus Reagents and size-selected with the Pippin Prep™ instrument

Figure 8 Bioanalyzer® instrument analysis of an unamplified 200-base-read library

1 µg of *E. coli* DH10B DNA was fragmented with the Ion Shear™ Plus Reagents with a reaction time adjusted for 200-base-read libraries. An unamplified library was prepared from the fragmented DNA and size-selected with the Pippin Prep™ instrument for 200-base-read libraries (330-bp length), as described in this user guide. Analysis was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

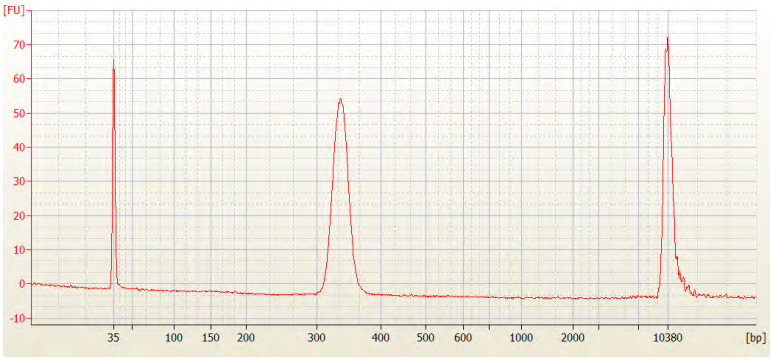
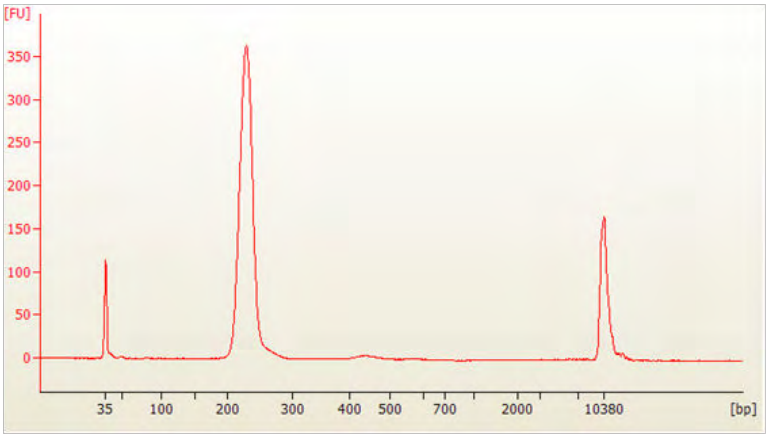


Figure 9 Bioanalyzer® instrument analysis of a 150-base-read library for the Ion Proton™ System

1 µg of human gDNA was fragmented with the Ion Shear™ Plus Reagents for 40 minutes. The fragmented DNA was size-selected with the Pippin Prep™ instrument for 150-base-read libraries (220-bp length) and then amplified, as described in this user guide. Analysis of a final library aliquot diluted 1:10 was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.



DNA fragmented with the Bioruptor® System and size-selected with the Pippin Prep™ instrument

Figure 10 Bioanalyzer® instrument analysis of a 400-base-read library

1 µg of *E. coli* DH10B DNA was fragmented with the Bioruptor® NGS System. The fragmented DNA was size-selected with the Pippin Prep™ instrument for 400-base-read libraries (490-bp length) using the 2% DF Marker L Agarose cassette and then amplified, as described in this user guide. Analysis of a final library aliquot diluted 1:10 was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

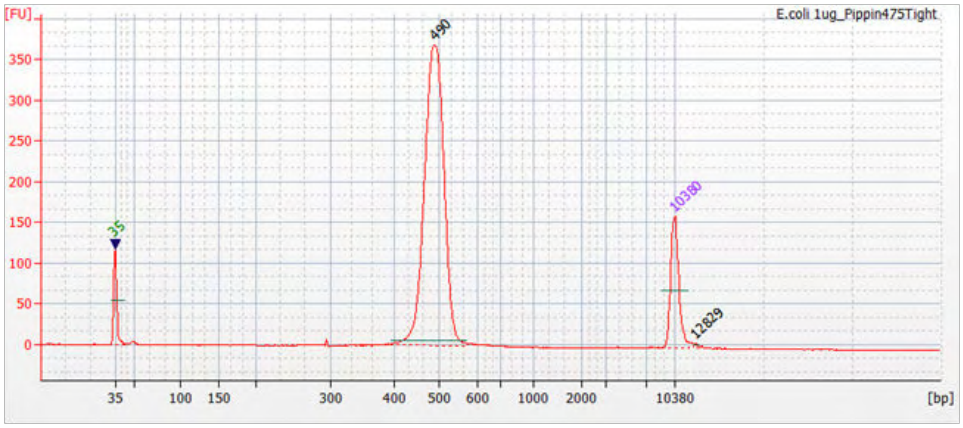


Figure 11 Bioanalyzer® instrument analysis of a 300-base-read library

1 µg of *E. coli* DH10B DNA was fragmented with the Bioruptor® NGS System for 20 minutes. The fragmented DNA was size-selected with the Pippin Prep™ instrument for 300-base-read libraries (390-bp length) and then amplified, as described in this user guide. Analysis of a final library aliquot diluted 1:10 was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

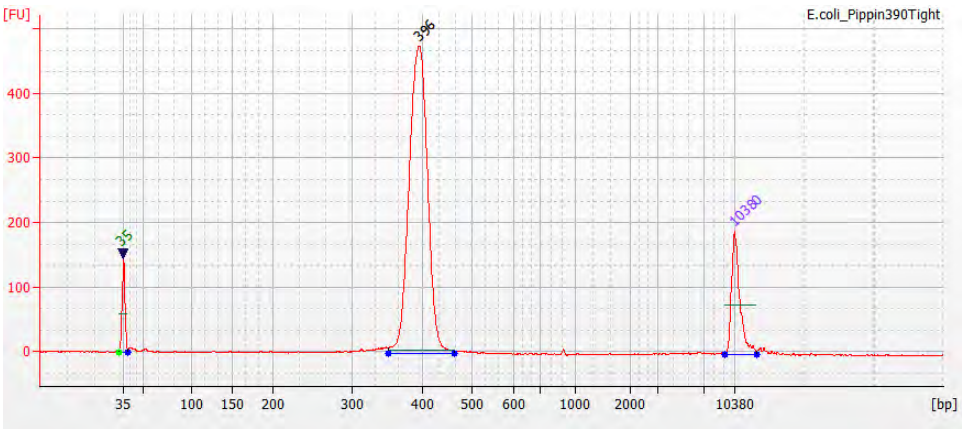


Figure 12 Bioanalyzer® instrument analysis of a 200-base-read library

1 µg of *E. coli* DH10B DNA was fragmented with the Bioruptor® NGS System for 17 minutes. The fragmented DNA was size-selected with the Pippin Prep™ instrument for 200-base-read libraries (330-bp length) and then amplified, as described in this user guide. Analysis of a final library aliquot diluted 1:10 was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

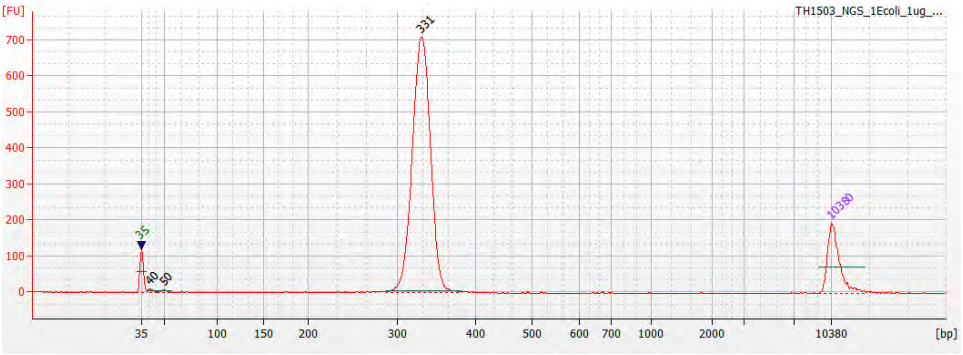
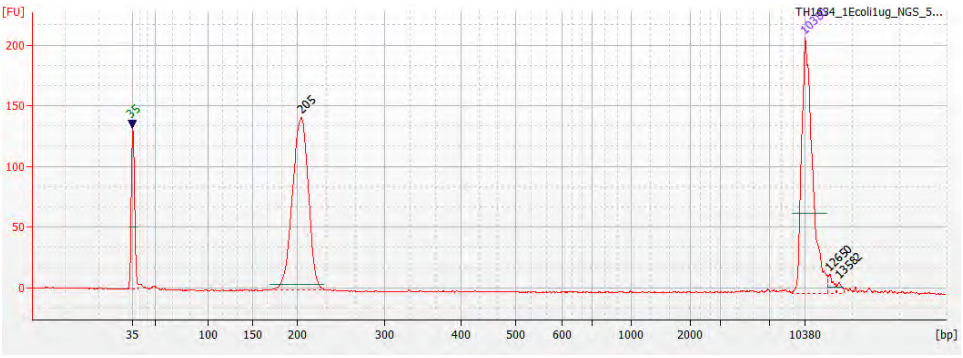


Figure 13 Bioanalyzer® instrument analysis of a 100-base-read library

1 µg of *E. coli* DH10B DNA was fragmented with the Bioruptor® NGS System for 17 minutes. The fragmented DNA was size-selected with the Pippin Prep™ instrument for 100-base-read libraries (200-bp length) and then amplified, as described in this user guide. Analysis of a final library aliquot diluted 1:10 was with the Agilent® High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.



Barcode discrimination

Torrent Suite™ Software v4.0.2 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)
5′-    CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′
3′-T*T*GGTAGAGTAGGGACGCACAGAGGCTGAGTC-5′
```

```
Ion P1 Adapter
5′-    CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGAT-3′
3′-T*T*GGTGATGCGGAGGCGAAAGGAGAGATACCCGTCAGCCACTA-5′
```

Barcode (A) adapter sequences

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

G

Safety

Chemical safety

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

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

Biological hazard safety

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

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**.

Note: For the SDSs of chemicals not distributed by Thermo Fisher Scientific, contact the chemical manufacturer.

Obtaining Certificates of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

Obtaining support

For the latest services and support information for all locations, go to:

www.lifetechnologies.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**ionsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Ion contact information

Web site: lifetechnologies.com/iontorrent

Ion community: ioncommunity.lifetechnologies.com

Support email: ionsupport@lifetech.com

Phone numbers
In North America: 1-87-SEQUENCE (1-877-378-3623)
Outside of North America: +1-203-458-8552

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.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.