iontorrent

Ion Xpress™ Plus gDNA Fragment Library Preparation

USER GUIDE

for use with:
Ion Plus Fragment Library Kit
Ion Plus Fragment Library Kit 48 rxns
Ion Xpress™ Plus Fragment Library Kit
Ion Xpress™ Barcode Adapters 1–96 Kit
Ion Plus Fragment Library Adapters
Ion Library Equalizer™ Kit

Catalog Numbers 4471252, A28950, 4471269, 4474517, 4476340, and 4482298

Publication Number MAN0009847

Revision K.0

ThermoFisher SCIENTIFIC

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

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

Revision history: Pub. No. MAN0009847

Revision	Date	Description of change
K.0	27 July 2020	• Added guidance for quantifying ≥300-bp libraries in "Pool barcoded libraries using qPCR (unamplified libraries or amplified libraries)" on page 77. • Added "Related documentation" on page 107
J.0	01 May 2020	Updated source information for Covaris™ products in "Required for physical fragmentation of gDNA" on page 16.
H.0	05 March 2019	Purification protocol for E-Gel™-size-selected libraries ("Purify the library (E-Gel™ SizeSelect™ II Agarose Gel size-selected libraries only)") moved from Chapter 6, "Size-select the library with the E-Gel™ SizeSelect™ II Agarose Gel" to Appendix A, "Equalize the library (for up to 300-base-read libraries)".
G.0	28 January 2019	• Replaced the discontinued E-Gel™ SizeSelect™ 2% Agarose Gel (Cat. No. G661002) with E-Gel™ SizeSelect™ II 2% Agarose Gel (Cat. No. G661012). • Replaced the discontinued E-Gel™ iBase™ and E-Gel™ Safe Imager™ Combo Kit with E-Gel™ Power Snap Electrophoresis Device. • Updated the protocol in Chapter 6, "Size-select the library with the E-Gel™ SizeSelect™ II Agarose Gel" to accommodate the E-Gel™ SizeSelect™ II 2% Agarose Gel and the E-Gel™ Power Snap Electrophoresis Device. • Purification protocol for E-Gel™-size-selected libraries moved from *Appendix A* to *Chapter 6*. • Updated sequencer information to include Ion GeneStudio™ S5 Series System. • Replaced the discontinued Ion 520™ & Ion 530™ Kit – Chef with Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef. • Removed the discontinued Ion PGM™ Hi-Q™ Sequencing Kit (Cat. No. A25592) • Added the recommended sequencing calibration standards in "Required materials not supplied" on page 15. • Removed the discontinued E-Gel™ SizeSelect™ 1.5% Agarose Gel (Cat. No. 4486125). • Removed the *Ion non-barcoded and barcode adapter sequences* appendix. • Moved the Bioruptor™ Sonication System physical fragmentation protocol from *Chapter 4* to *Appendix B*. • Updated the list of required materials for physical fragmentation procedures using the Covaris™ sonication systems in *Chapter 1* and *Chapter 4*.
F.0	10 November 2016	• Support added for preparing 600-base-read libraries.
E.0	15 July 2016	• Ordering changes for the Pippin Prep™ instrument and related kits
D.0	26 April 2016	• Rebranding • Added 48 reaction Ion Plus Fragment Library Kit • Added support for preparing 500-base-read libraries for templating with the Ion PGM™ Template IA 500 Kit • Chapter reorganization
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	03 March 2014	• Changed the recommendation for final library dilution to 100 pM to unify the kit with other Ion library and template kits.

Contents

1 Product information

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

Product description

Products covered by this guide

- Ion Plus Fragment Library Kit (Cat. No. 4471252)
- Ion Plus Fragment Library Kit 48 rxns (Cat. No. A28950)
- Ion Xpress™ Plus Fragment Library Kit (Cat. No. 4471269)–includes the components from the following kits:
  - Ion Plus Fragment Library Kit
  - Ion Shear™ Plus Reagents Kit
- Ion Xpress™ Barcode Adapters Kit (Cat. No. 4474517)
- Ion Plus Fragment Library Adapters (Cat. No. 4476340)
- Ion Library Equalizer™ Kit (Cat. No. 4482298)

The Ion Plus Fragment Library Kit (Cat. No. 4471252, A28950) and the Ion Xpress™ Plus Fragment Library Kit (Cat. No. 4471269) are used to prepare fragment libraries from genomic DNA (gDNA) for downstream template preparation and sequencing on an Ion PGM™ Sequencer, Ion Proton™ Sequencer, Ion S5™ Sequencer, Ion S5™ XL Sequencer, or Ion GeneStudio™ S5 Series Sequencer.

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

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

Template kit compatibility

The library kits listed above are compatible with all current Ion Torrent™-branded template preparation kits for the Ion PGM™ System, Ion Proton™ System, Ion S5™ System, Ion S5™ XL System, or Ion GeneStudio™ S5 Series System.

IMPORTANT!

- For optimal performance of the Ion PGM™ Template IA 500 Kit (Cat. No. A24622), we recommend for 400–500-base-read libraries to be size-selected using the Pippin Prep™ instrument and quantified with the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802).
- 600-base-read libraries can only be used with the Ion 520™ & Ion 530™ ExT Kit – Chef (Cat. No. A30670) for sequencing on the Ion S5™ System, Ion S5™ XL System, or Ion GeneStudio™ S5 Series System. For more information, see the *Ion 520™ & Ion 530™ ExT Kit – Chef User Guide* (Pub. No. MAN0015805).

Sequencing system	Library size	Template kits
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System	600-base-read	Ion 520™ & Ion 530™ ExT Kit – Chef
	500-base-read	
	400-base-read	Ion 520™ & Ion 530™ ExT Kit – Chef Ion 520™ & Ion 530™ Kit – OT2 Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef
	300-base-read	Ion 520™ & Ion 530™ Kit – OT2 Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef
	200-base-read	Ion 520™ & Ion 530™ Kit – OT2 Ion 540™ Kit – OT2 Ion 510™ & Ion 520™ & Ion 530™ Kit – Chef Ion 540™ Kit – Chef Ion 550™ Kit – Chef
	100-base-read	
Ion PGM™ System	500-base-read	Ion PGM™ Template IA 500 Kit
	400-base-read	Ion PGM™ Hi-Q™ View OT2 Kit Ion PGM™ Hi-Q™ View Chef Kit Ion PGM™ Hi-Q™ View Chef 400 Kit
	300-base-read	
	200-base-read	
	100-base-read	

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Sequencing system	Library size	Template kits
Ion Proton™ System	200-base-read	Ion PI™ Hi-Q™ OT2 200 Kit Ion PI™ Hi-Q™ Chef Kit
	150-base-read	Ion PI™ Hi-Q™ OT2 200 Kit Ion PI™ Hi-Q™ Chef Kit

Contents and storage

Ion Plus Fragment Library Kit

Use the Ion Plus Fragment Library Kit (Cat. No. 4471252), or the Ion Plus Fragment Library Kit 48 rxns (Cat. No. A28950), to prepare libraries from enzymatically or physically fragmented genomic DNA (gDNA) for downstream template preparation and sequencing on an Ion PGM™ Sequencer, Ion Proton™ Sequencer, Ion S5™ Sequencer, Ion S5™ XL Sequencer, or Ion GeneStudio™ S5 Series Sequencer. The Ion Plus Fragment Library Kit is compatible with all current Ion Torrent™-branded template preparation kits for these systems. This kit also includes reagents for end-repair of physically fragmented gDNA and reagents for library preparation from the end-repaired DNA.

- Ion Plus Fragment Library Kit (Cat. No. 4471252) provides reagents for preparing up to 20 libraries at 100-ng input, or up to 10 libraries at 1-µg input.
- Ion Plus Fragment Library Kit 48 rxns (Cat. No. A28950) provides reagents for preparing up to 96 libraries at 100-ng input, or up to 48 libraries at 1-µg input.

Contents	Cap color	Amount		Storage
		4471252 (10 reactions)	A28950 (48 reactions)	
5X End Repair Buffer[1]	Red	400 µL	1.92 mL	-30°C to -10°C
End Repair Enzyme[1]	Orange	20 µL	96 µL	
10X Ligase Buffer	Yellow	200 µL	960 µL	
DNA Ligase	Blue	40 µL	192 µL	
Nick Repair Polymerase	Clear	160 µL	768 µL	
dNTP Mix	Violet	40 µL	192 µL	
Adapters	Green	100 µL	480 µL	
Platinum™ PCR SuperMix High Fidelity	Black/—	2 × 1000 µL	9.6 mL	
Library Amplification Primer Mix	White	100 µL	480 µL	
Low TE	Clear/—	2 × 1.5 mL	14.4 mL	15°C to 30°C [2]

[1] Required only for physically fragmented gDNA.

[2] Low TE can also be stored at -30°C to -10°C for convenience

Ion Xpress™ Plus Fragment Library Kit

The Ion Xpress™ Plus Fragment Library Kit (4471269) is used to prepare fragment libraries from genomic DNA (gDNA) for downstream template preparation and sequencing on the Ion PGM™ Sequencer, Ion Proton™ Sequencer, Ion S5™ Sequencer, Ion S5™ XL Sequencer, or Ion GeneStudio™ S5 Series Sequencer .

Ion Xpress™ Plus Fragment Library Kit provides reagents for preparing up to 20 libraries at 100-ng input, or up to 10 libraries at 1 µg input, and includes the components from the following kits:

- Ion Plus Fragment Library Kit: used for library preparation from the enzymatically fragmented DNA (see "Ion Plus Fragment Library Kit" on page 12 for kit components).
- Ion Shear™ Plus Reagents Kit: used for enzymatic fragmentation of gDNA (see the following table for kit components).

Contents	Cap color	Amount	Storage
Ion Shear™ Plus Enzyme Mix II [1]	Clear	2 × 100 µL	–30°C to –10°C
Ion Shear™ Plus 10X Reaction Buffer	Clear	2 × 50 µL	
Ion Shear™ Plus Stop Buffer	Clear	2 × 50 µL	

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

Ion Xpress™ Barcode Adapters Kits

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.

The following Ion Xpress™ Barcode Adapters Kits are available:

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

Contents	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 tube per barcode)	20 µL each	

[1] X = barcode number

Ion Plus Fragment Library Adapters

The Ion Plus Fragment Library Adapters (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 covered by this user guide. The kit contains the following components:

Contents	Cap color	Amount	Storage
Adapters	Green	100 µL	–30°C to –10°C
Library Amplification Primer Mix	White	100 µL	

Ion Library Equalizer™ Kit

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

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

Contents	Cap color	Amount	Storage[1]
Equalizer™ Primers	Red	200 µL	2°C to 8°C
Equalizer™ Capture	Purple	1 mL	
Equalizer™ Elution Buffer	—	10 mL	
Equalizer™ Beads	Orange	300 µL	
Equalizer™ Wash Buffer	—	35 mL	15°C to 30°C

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

Required materials not supplied

Required for all types of library preparation

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

Item	Source
Instruments and Equipment:	
Agilent™ 2100 Bioanalyzer™ instrument	G2939BA
Qubit™ Fluorometer[1]	Q33226
DynaMag™-2 Magnet (magnetic rack)	12321D
Microcentrifuge	MLS
Thermal cycler	MLS
Vortex mixer	MLS
Pipettors 1–1000 µL	MLS
Reagents and consumables:	
Agencourt™ AMPure™ XP Kit	Beckman Coulter™ A63880
Agilent™ High Sensitivity DNA Kit	Agilent™ 5067-4626
Qubit™ dsDNA HS Assay Kit	Q32854
Nuclease-free Water (not DEPC-Treated)	AM9932
(Optional) Ion Library TaqMan™ Quantitation Kit[2] (required for quantification of unamplified libraries)	4468802
(Optional) Ambion™ RNase I, cloned, 100 U/µL	AM2294
(Optional) PureLink™ Genomic DNA Mini Kit (for cleanup after optional RNase treatment)	K1820-01
(Optional) 10 mM Tris, pH 7.5–8.5	MLS
Qubit™ Assay Tubes	Q32856
Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5-mL	Fisher Scientific™ 13-698-791 Eppendorf™ 022431021
PCR tubes, 0.2-mL	MLS
Barrier pipette tips	MLS

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Item	Source
***(Optional)* One of the following library preparation and templating controls:**	
Ion S5™ Controls Kit Plus	A30729
Ion PGM™ Controls Kit v3	A30046
Ion PI™ Controls 200 Kit	4488985
E. coli DH10B Control 600 Library	A32470
***(Optional)* One of the following calibration standards for *de novo* sequencing applications:**	
Ion PGM™ Calibration Standard	A27832
Ion S5™ Calibration Standard	A27988
Ion S5™ ExT Calibration Standard	A30738

[1] Qubit™ 2.0 Fluorometer or later.
[2] Not compatible with 600-base-read libraries.

Required for physical fragmentation of gDNA

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

Item	Source
One of the following sonication systems:	
Covaris™ M220 Focused-ultrasonicator™ Instrument (110 V for U.S. customers; 220 V for international customers)	Covaris 500295
Covaris™ S220 Focused-ultrasonicator (110 V for U.S. customers; 220 V for international customers)	Covaris 500217
M220 supplies:	
Covaris™ microTUBE™-50 AFA Fiber Screw-Cap tubes *or* Covaris™ microTUBE™ AFA Fiber Snap-Cap tubes	Covaris
Covaris™ M220 Holder XTU	
Covaris™ M220 Holder Insert microTUBE™ (50 µL or 130 µL)	
Covaris™ microTUBE™ Prep Station Snap&Screw Cap	
Covaris™ M220 AFA-grade Water[1]	

(continued)

Item	Source
S220 supplies:	
Covaris™ microTUBE™ AFA Fiber Snap-Cap tubes	Covaris
Covaris™ S-Series Holder microTUBE™	
Covaris™ microTUBE™ Prep Station Snap&Screw Cap	
Ethylene glycol	MLS

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

Use the following table to determine which physical fragmentation system is appropriate for your experiment:

Library size	Sonication system	Protocol
100–600-base-read	Covaris™ M220	"Fragment gDNA with the Covaris™ M220 Focused-ultrasonicator™ Instrument" on page 32
600-base-read	Covaris™ S220 Covaris™ S2 [1]	"Fragment gDNA with the Covaris™ S2 and S220 Systems" on page 35
100–500-base-read	Bioruptor™ CD-200TS[1] Bioruptor™ UCD-600 NGS[1]	Appendix B, "Prepare adapter-compatible DNA: physical fragmentation with the Bioruptor™ Sonication System"

[1] Discontinued, but supported. Contact manufacturer for details.

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

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

Note: The following items can also be ordered as part of the E-Gel™ Power Snap Electrophoresis Device Starter Kit, Size Select II 2% (Cat. No. G8162ST).

Item	Source
E-Gel™ Power Snap Electrophoresis Device[1]	G8100
(Optional) E-Gel™ Power Snap Camera	G8200
E-Gel™ SizeSelect™ II Agarose Gel, 2%	G661012
E-Gel™ Sizing DNA Ladder	10488100
UltraPure™ DNase/RNase-Free Distilled Water	10977015, 10977023

[1] Replaced the E-Gel™ iBase™ and E-Gel™ Safe Imager™ Combo Kit (Cat. No. G6465). For more information, contact Technical Support.

Required for size-selection using the Pippin Prep™ System

The Pippin Prep™ DNA Size Selection System and associated kits can be purchased from Sage Science (**http://www.sagescience.com/**).

Item	Source
Pippin Prep™ DNA Size Selection System	PIP0001
Pippin Prep™ Gel Cassette with ethidium bromide, 2% Agarose with external markers, 100–600 bp, 10/pkg	CSD2010
Pippin Prep™ Gel Cassette, dye free, 2% Agarose with internal standards, 100–600 bp, 10/pkg	CDF2010
Pippin Prep™ Gel Cassette, dye free, 1.5% Agarose with internal standards, 250 bp–1.5 kb, 10/pkg	CDF1510

2 Procedural guidelines and workflow overview

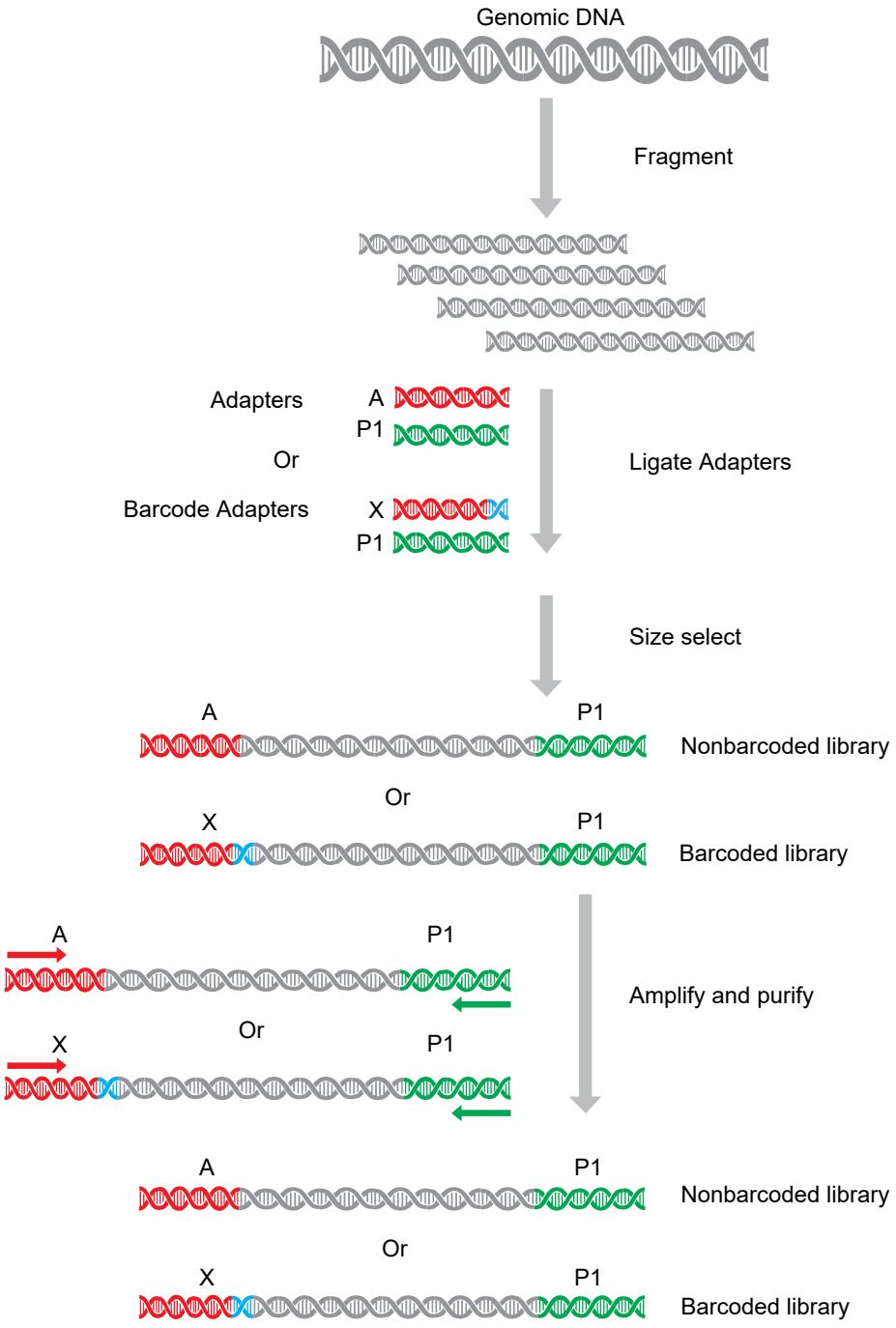
Procedural guidelines

IMPORTANT! High-quality RNA-free DNA is required for this procedure. The quality of the input DNA has a significant impact on the quality of the resulting library. Several commercially available kits are available for isolation of high molecular weight, RNA-free genomic DNA. For more information on the assessment of the integrity and size of input DNA and the optional RNase treatment procedure, see Appendix C, “Evaluate the quality of the genomic DNA”.

- Use good laboratory practices to minimize cross-contamination of products. If possible, perform library construction in an area or room that is separate from template preparation.
- When handling barcoded adapters, be especially careful not to cross-contaminate. Change gloves frequently and open one tube at a time.
- For all steps that require 1.5-mL tubes, use the 1.5-mL Eppendorf LoBind™ tubes.
- Thaw reagents on ice before use; keep enzymes at –30°C to –10°C until ready to use.
- Mix reagents thoroughly before use, especially if frozen and thawed.
- Use the Agencourt™ AMPure™ XP Kit for DNA purification. Use the Agilent™ 2100 Bioanalyzer™ instrument to analyze DNA fragment length distribution during library preparation.

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

Select the workflow options that are appropriate for your experimental needs and lab setup.

Fragmentation options to prepare adapter-compatible DNA (30–120 minutes)		
Method	**Ion Xpress™ Plus Fragment Library Kit** (Chapter 3, "Prepare adapter-compatible DNA: fragment gDNA with Ion Xpress™ Plus Fragment Library Kit"	**Bioruptor™ Sonication System** (Chapter 4, "Prepare adapter-compatible DNA: physical fragmentation")
Input amount	50–100 ng or 1 µg gDNA	100 ng or 1 µg gDNA
Library Size[1]	100–600-base-read libraries	100–500-base-read libraries
Features	Can customize fragmentation profile by adjusting reaction time No end-repair required	Same fragmentation conditions for 200-base-read and 100-base-read libraries using the Bioruptor™ NGS UCD-600
Time	Ion PGM™ System, Ion Proton™ System, Ion S5™ System, Ion S5™ XL System, or Ion GeneStudio™ S5 Series System: 300-base-read and above libraries: ~30 minutes 200-base-read libraries: ~40 minutes 100-base-read libraries: ~60 minutes	55–120 minutes depending on the library and instrument used
	Ion Proton™ System: 200-base-read libraries: ~50 minutes 150-base-read libraries: ~60 minutes	

[1] Includes 150- and 200-base-read libraries for the Ion Proton™ System. Sequence 100–600-base-read libraries on the Ion PGM™ Sequencer, Ion Proton™ Sequencer, Ion S5™ Sequencer, Ion S5™ XL Sequencer, or Ion GeneStudio™ S5 Series Sequencer.

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Adaptor ligation, nick-repair, and purification (~40 minutes)

▼

Size-selection options (20–90 minutes)		
Method	**2% E-Gel™ SizeSelect™ II Agarose Gel** (Chapter 6, "Size-select the library with the E-Gel™ SizeSelect™ II Agarose Gel")	**Pippin Prep™ instrument** (Chapter 7, "Size-select the library with the Pippin Prep™ System")
Features	• Faster • Broader size distribution	• Automated • Tighter size distribution results in more consistent library size • Preferred for 500-base-fragment size selection
Time	500–600-base-read libraries: ~40 minutes 400-base-read libraries: ~35 minutes 300-base-read libraries: ~30 minutes 100–200-base-read libraries: ~20 minutes	90 minutes

▼

Library normalization/quantification options[1] (40–70 minutes)			
Method	**Ion Library Equalizer™ Kit** (Appendix A, "Equalize the library (for up to 300-base-read libraries)"	**qPCR** (Chapter 8, "Amplify and purify the library")	**Bioanalyzer™ instrument analysis** (Appendix D, "Example Bioanalyzer™ traces")
Features	No library quantification required	Amplification optional[1]	Amplification optional[1]
Time	~40 minutes	~70 minutes	~60 minutes

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

3 Prepare adapter-compatible DNA: fragment gDNA with Ion Xpress™ Plus Fragment Library Kit

This chapter describes conditions for enzymatic fragmentation of gDNA into blunt-ended fragments, using the Ion Xpress™ Plus Fragment Library Kit. This method is suitable for 100–600-base-read libraries.

Following shearing, the fragmented DNA is ready for adapter ligation. No end-repair is required.

Note: For physical fragmentation methods, see Chapter 4, “Prepare adapter-compatible DNA: physical fragmentation”.

Procedural guidelines

- *(FFPE samples only)* 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.
- The Ion Shear™ reaction has good tolerance of the G+C content of a sample. However, the Ion Shear™ reaction is sensitive to EDTA concentration, the integrity of the sample, and operator handling method. For 1 µg-input samples, ensure that the reaction time is optimal for your laboratory conditions.

IMPORTANT! We recommend for the first-time users to prepare a library using control samples before using their own samples, to familiarize themselves with the fragmentation and library preparation procedures. The following table lists genomic DNA controls found in control kits for use with Ion Torrent™-branded sequencing systems.

Control DNA	System
Ion S5™ Controls Kit Plus (Cat. No. A30729)	
Human CEPH Genomic DNA Control	Ion S5™ System, Ion S5™ XL System, or Ion GeneStudio™ S5 Series System
Ion PGM™ Controls Kit v3 (Cat. No. A30046)	
E. coli DH10B Genomic DNA Control	Ion PGM™ System
Ion PI™ Controls 200 Kit (Cat. No. 4488985)	
Human CEPH Genomic DNA Control	Ion Proton™ System

Input DNA

Prepare high-quality, RNA-free genomic DNA (gDNA) using commercially available kits (see Appendix C, "Evaluate the quality of the genomic DNA"). The recommended input is 1 µg of gDNA per library. 50–100 ng of high-quality gDNA per library has been successfully used with this protocol.

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

Library and fragment sizes

The Ion Shear™ fragmentation method is suitable for 100–600-base-read libraries. Select the fragmentation conditions according to the desired library size.

Sequencing system	Library size	Median fragment size
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System	600-base-read	550–650 bp
	500-base-read	470-570 bp
	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 PGM™ System	500-base-read	470-570 bp
	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 required

The following materials are 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:

- 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 Reagent
- Freshly prepared 70% ethanol
- Magnetic rack
- *(Optional)* Control DNA
  - *E. coli* DH10B Genomic Control
  - Human CEPH Genomic 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 needed, 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:

- If you are running a control reaction, prepare a control sample in a separate tube.
- For the control reaction, mix 1 µL (100 ng) of *E. coli* DH10B Genomic DNA Control or Human CEPH Genomic DNA Control with 9 µL of Nuclease-free Water or 10 mM Tris, pH 7.5–8.5. Follow the standard protocol for a 100-ng sample.

1. Determine the volume of input gDNA, then adjust the concentration as necessary.

Input amount	Procedure
50–100 ng	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.
1 µg	Prepare 10 µL at 100 ng/µL in Nuclease-free Water or 10 mM Tris, pH 7.5–8.5.

2. Vortex the Ion Shear™ Plus 10X Reaction Buffer and the Ion Shear™ Plus Enzyme Mix II each for 5 seconds. Pulse-centrifuge to bring the contents to the bottom of the tubes, then 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, then mix vigorously by vortexing for 5 seconds. Pulse-centrifuge 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	
	50–100 ng DNA input	1 µg DNA input
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.

IMPORTANT! Do not mix by vortexing. Avoid creating bubbles.

6. Incubate the tubes in a water bath or heat block at 37°C for the indicated reaction time.

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

Median fragment size	Reaction time	Optimization range
550–650 bp	6 minutes	4–10 minutes
470–570 bp	7 minutes	4–12 minutes
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, then 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 this procedure. A higher percentage of ethanol causes inefficient washing of smaller-sized molecules. A lower percentage of ethanol can cause sample loss.

1. Add Agencourt™ AMPure™ XP Reagent as described in the following table:

Option	Description
600-base-read library	Add 55 µL of Agencourt™ AMPure™ XP Reagent (1X sample volume)
500-base-read and smaller libraries	Add 99 µL of Agencourt™ AMPure™ XP Reagent (1.8X sample volume)

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

3. Pulse-centrifuge, then 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, then discard the supernatant without disturbing the bead pellet.

4. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol. Incubate for 30 seconds, turning the tube 3-4 times in the magnet to agitate the beads. After the solution clears, remove, then discard the supernatant without disturbing the pellet.

5. Repeat step 4 one more time.

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

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

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

9. Pulse-centrifuge, then place the tube in the magnetic rack for at least 1 minute or until the solution clears. Transfer the supernatant containing the eluted DNA to a new 0.2-mL PCR tube without disturbing the pellet.

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

10. *(Optional)* Check the fragment size using the Agilent™ 2100 Bioanalyzer™ instrument and Agilent™ High Sensitivity DNA Kit. See the following table to determine the volume to use, depending on the amount of input DNA.

Input	Volume
50–100 ng	1 µL of 1:5 dilution [1]
1 µg	1 µL of 1:10 dilution [1]

[1] Prepare the dilution in Low TE.

11. Confirm the desired DNA fragment size range.

Sequencing system	Library type	Target median fragment size	Fragment size range
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System	600-base-read library	550–650 bp	200–1200 bp
	500-base-read library	470–570 bp	150–1000 bp
	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 PGM™ System	500-base-read library	470–570 bp	150–1000 bp
	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

(continued)

Sequencing system	Library type	Target median fragment size	Fragment size range
Ion Proton™ System	200-base-read library	150–250 bp	100–700 bp
	150-base-read library	100–200 bp	50–500 bp

Note: For example traces, see Figures 1–3 in Appendix D, "Example Bioanalyzer™ traces".

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

Proceed to Chapter 5, "Ligate adapters, nick-repair, and purify the ligated DNA".

4 Prepare adapter-compatible DNA: physical fragmentation

This chapter describes conditions for physically shearing genomic DNA with the Covaris™ sonication systems. The sonicator generates DNA fragments suitable for preparing 100–600-base-read libraries for use with Ion Torrent™-branded sequencing systems, including the 150- and 200-base-read libraries for the Ion Proton™ System. The sonicated DNA is ready for end-repair. After fragmentation, prepare the libraries by adjusting the downstream size-selection of the library molecules.

Note: If you have the Bioruptor™ Sonication System, see Appendix B, "Prepare adapter-compatible DNA: physical fragmentation with the Bioruptor™ Sonication System".

Library size	Sonication system	Protocol
100–600-base-read	Covaris™ M220	"Fragment gDNA with the Covaris™ M220 Focused-ultrasonicator™ Instrument" on page 32
600-base-read	Covaris™ S220 Covaris™ S2 [1]	"Fragment gDNA with the Covaris™ S2 and S220 Systems" on page 35
Other protocols:		
100–500-base-read	Bioruptor™ CD-200TS[1] Bioruptor™ UCD-600 NGS[1]	Appendix B, "Prepare adapter-compatible DNA: physical fragmentation with the Bioruptor™ Sonication System"

[1] Discontinued, but supported.

Note: For fragmentation using the Ion Xpress™ Plus Fragment Library Kit, see Chapter 3, "Prepare adapter-compatible DNA: fragment gDNA with Ion Xpress™ Plus Fragment Library Kit".

Prepare genomic DNA

Prepare high-quality, RNA-free genomic DNA (gDNA) using one of the commercially available kits (see Appendix C, "Evaluate the quality of the genomic DNA"). Although the recommended input is 1 µg of gDNA per library, 50–100 ng of high-quality gDNA per library has been successfully used with this protocol.

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

IMPORTANT! We recommend for the first-time users to prepare a library using control samples before using their own samples, to familiarize themselves with the fragmentation and library preparation procedures. The following table lists genomic DNA controls that are included in control kits for use with Ion Torrent™-branded sequencing systems.

Control DNA	System
Ion S5™ Controls Kit Plus (Cat. No. A30729)	
Human CEPH Genomic DNA Control	Ion S5™ System, Ion S5™ XL System, or Ion GeneStudio™ S5 Series System
Ion PGM™ Controls Kit v3 (Cat. No. A30046)	
E. coli DH10B Genomic DNA Control	Ion PGM™ System
Ion PI™ Controls 200 Kit (Cat. No. 4488985)	
Human CEPH Genomic DNA Control	Ion Proton™ System

Fragment gDNA with the Covaris™ M220 Focused-ultrasonicator™ Instrument

This section describes shearing of gDNA with the Covaris™ M220 Focused-ultrasonicator™ Instrument to generate DNA fragments that are appropriate for 100–600-base-read libraries for sequencing on the Ion S5™ System, Ion S5™ XL System, Ion GeneStudio™ S5 Series System, or Ion PGM™ System.

For additional instructions on using the Covaris™ M220 Focused-ultrasonicator™ Instrument, including loading and unloading the Covaris™ microTUBE™ tubes in the Covaris™ M220 Holder XTU, contact the manufacturer (**www.covarisinc.com**).

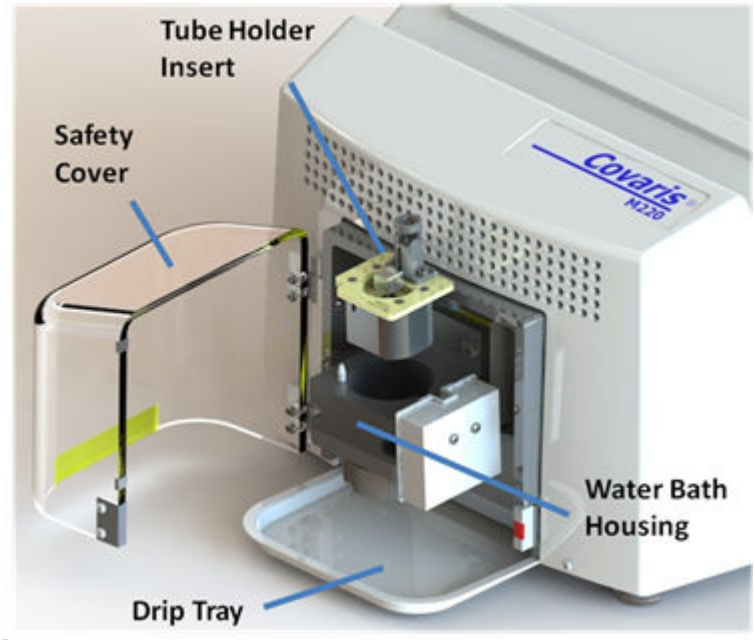
IMPORTANT! The parameters for fragmenting 500–600-base-read libraries are recommendations and can require optimization. For more information, consult the manufacturer.

Materials required

- Covaris™ M220 Focused-ultrasonicator™ Instrument
- SonoLab 7 Software
- Covaris™ microTUBE™-50 AFA Fiber Screw-Cap tubes
- *(Required for 500–600-base-read libraries)* Covaris™ microTUBE™ AFA Fiber Snap-Cap tubes
- Covaris™ M220 Holder XTU with the Covaris™ M220 Holder Insert microTUBE™ (50 µL or 130 µL)
- Covaris™ microTUBE™ Prep Station Snap&Screw Cap
- Covaris™ M220 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; from Ion Plus Fragment Library Kit)
- Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5-mL

Note: Settings for processing 50-µL samples in Covaris™ microTUBE™-50 AFA Fiber Screw-Cap tubes are available on the Covaris™ website: **http://covarisinc.com/products/afa-ultrasonication/m-series/m220-ion-compatible/**

Procedure



1. Open the safety cover, then place the Tube Holder (Covaris™ M220 Holder XTU) into the water bath housing.

2. Fill the Covaris™ water bath housing with Covaris™ M220 AFA-grade Water using the provided wash bottle. Continue adding water until the water reaches the top surface of the Tube Holder (~15 mL) and the water level indicator in the SonoLab 7 Software turns to green.

3. Dilute DNA in an 1.5-mL Eppendorf LoBind™ tube:

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

4. Place a Covaris™ microTUBE™ tube into the Prep Station (Covaris™ microTUBE™ Prep Station Snap&Screw Cap). Remove the cap, then use a tapered pipette tip to slowly transfer the diluted DNA sample into the Covaris™ microTUBE™ tube. Close the Covaris™ microTUBE™ tube.

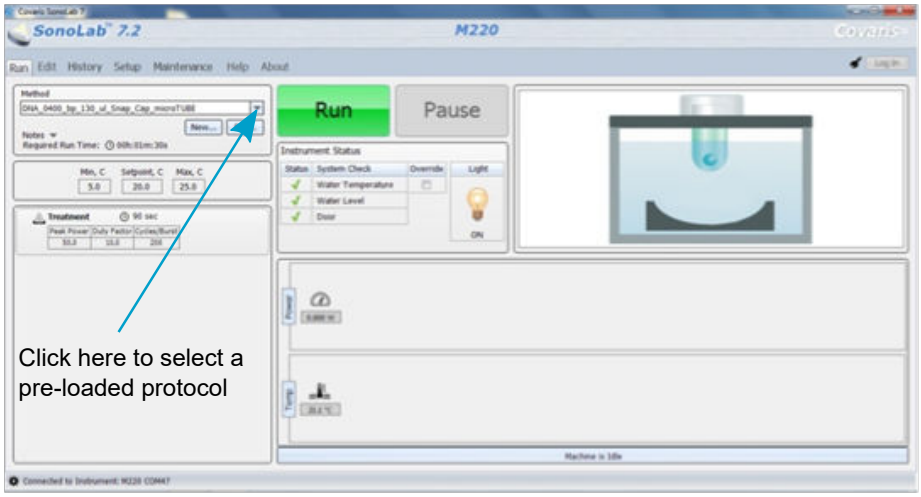
   Note: Be careful not to introduce a bubble into the bottom of the tube.

5. Select a pre-loaded protocol or enter settings manually in the SonoLab software according to the library size.

   - *(100–400-base-read libraries)* Select the pre-loaded protocol in the SonoLab software:

     Note: Temperature is preprogrammed for each protocol and will be automatically regulated by the M220 after the protocol is 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



- Enter settings manually:

Library size	Mean fragment size	Peak Incident Power (PIP)	Duty Factor	Cycles per burst	Treatment time (seconds)	Temp	Sample volume
600 bp	ND[1,2]	50 W	7.5%[2]	200	51[2]	20°C	130 µL
500 bp	ND[1,2]	50 W	10%[2]	200	50[2]	20°C	130 µL
400 bp	410 bp	50 W	20%	200	60	20°C	50 µL
300 bp	320 bp	50 W	20%	200	100	20°C	50 µL
200 bp	260 bp	50 W	20%	200	130	20°C	50 µL
100 bp	150 bp	50 W	20%	200	375	20°C	50 µL

[1] Not Determined.

[2] Recommendations only. Likely will require optimization. For more information, contact the manufacturer.

6. Place the Covaris™ microTUBE™ tube into the Tube Holder, close the Safety Cover, then click **Run** in the SonoLab software.

7. After the treatment is finished, place the Covaris™ microTUBE™ tube into the Prep Station. Unscrew the cap, slowly remove the sheared DNA, then transfer the DNA into a new 1.5-mL Eppendorf LoBind™ tube.

Fragment gDNA with the Covaris™ S2 and S220 Systems

This section describes sonication of gDNA with the Covaris™ S2 and S220 Systems to generate DNA fragments that are appropriate for 100–600-base-read libraries for sequencing on the Ion S5™ System, Ion S5™ XL System, Ion GeneStudio™ S5 Series System, or Ion PGM™ System. The sonicated DNA is ready for end-repair. For additional instructions on using the Covaris™ System, including loading and unloading the Covaris™ microTUBE™ AFA Fiber Snap-Cap tubes in the Covaris™ S-Series Holder microTUBE™, contact the manufacturer (**www.covarisinc.com**).

Materials required

- Covaris™ S2 or S220 System (110 V for U.S. customers; 220 V for international customers)
- Covaris™ microTUBE™ AFA Fiber Snap-Cap tubes
- Covaris™ S-Series Holder microTUBE™
- Covaris™ microTUBE™ Prep Station Snap&Screw Cap
- Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5-mL
- Low TE (10 mM Tris pH 8.0, 0.1 mM EDTA; from Ion Plus Fragment Library Kit)
- Ethylene glycol

Procedure

IMPORTANT! Set the chiller temperature to 2°C–5°C to ensure that the temperature reading in the water bath displays 5°C. Supplement the circulated water chiller, not the water bath itself, with 20% ethylene glycol.

1. Fill the Covaris™ water bath to level 12, then degas the water bath for 30 minutes before shearing.

   Note: 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 submerged completely.

2. Dilute DNA in a 1.5-mL Eppendorf LoBind™ tube.

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

3. Place a Covaris™ microTUBE™ AFA Fiber Snap-Cap tube into the loading station.

4. Keeping the cap on the tube, use a tapered pipette tip to slowly transfer the diluted DNA sample through the pre-split septa.

   Note: Be careful not to introduce a bubble into the bottom of the tube.

5. Shear the DNA using the following shearing conditions:

Condition	100–250-bp fragments	350-bp fragments	400-bp fragments	500-bp fragments
Covaris™ S220 System settings[1]				
Bath temperature	5°C	5°C	5°C	5°C
Duty cycle	10%	10%	10%	10%
Peak Incident Power (PIP)	175 W	175 W	175 W	175 W
Cycles/burst	100	100	100	100
Treatment time	360 seconds	90 seconds	80 seconds	60 seconds

[1] For information on system settings for 600-base-read libraries, consult Covaris, Inc. (**www.covarisinc.com**).

Condition	100–250-bp fragments	350-bp fragments	400-bp fragments	500-bp fragments	600-bp fragments
Covaris™ S2 System settings					
Number of cycles	6	2	2	2	2
Bath temperature	5°C	5°C	5°C	5°C	5°C
Bath temperature limit	12°C	10°C	10°C	10°C	10°C
Mode	Frequency sweeping	Frequency sweeping	Frequency sweeping	Frequency sweeping	Frequency sweeping
Water quality testing function	Off	Off	Off	Off	Off
Duty cycle	10%	10%	10%	10%	5%
Intensity	5	5	5	5	3
Cycles/burst	100	100	100	100	200
Treatment time	60 seconds	45 seconds	40 seconds	30 seconds	45 seconds

6. Place the Covaris™ microTUBE™ AFA Fiber Snap-Cap tube into the loading station.

7. Keeping the snap-cap on, insert a pipette tip through the pre-split septa, slowly remove the sheared DNA, then transfer the DNA into a new 1.5-mL Eppendorf LoBind™ tube.

8. Analyze the fragmented DNA using one of the following methods.

   - Dilute an aliquot 1:50 in low TE, then analyze DNA on the Agilent™ 2100 Bioanalyzer™ instrument using Agilent™ High Sensitivity DNA Kit.
   - Analyze an undiluted aliquot on an agarose gel.

Table 1 Bioanalyzer™ instrument peaks

Fragment range	Peak
100–250-bp	200 bp
350-bp	320 bp
400-bp	410 bp
500-bp	475–525 bp
600-bp	575–625 bp

9. 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 needed. If the volume of sheared DNA is >80 µL, concentrate the DNA to ~80 µL using a SpeedVac™ concentrator. If a SpeedVac™ concentrator is not available, perform an Agencourt™ AMPure™ XP Reagent purification step (see "Purify the fragmented DNA" on page 28). Use 1.8X sample volume of the AMPure™ XP Reagent, then elute the DNA in a volume of ~80 µL. Alternatively, use the end-repair reaction conditions for 1-µg input DNA, which can accommodate a 158-µL volume of DNA.

10. Proceed immediately to "End-repair and purify DNA" on page 38.

Assess the fragmentation profile

Analyze an aliquot of the fragmented DNA to confirm the presence of a fragment with a peak around 150 bp for 100-base-read libraries, 260 bp for 200-base-read libraries, 320 bp for 300-base-read libraries, and 410 bp for 400-base-read libraries. For example traces, see Figures 4-6 in Appendix D, "Example Bioanalyzer™ traces".

Note: For 500–600-base-read libraries, use the Agilent™ 2100 Bioanalyzer™ instrument to check the profile. If the profile is not accurate, contact Covaris, Inc. for recommendations on optimization (**www.covarisinc.com**).

Table 2 Recommended aliquot volume to use for fragment size analysis

Input DNA	Agilent™ High Sensitivity DNA Kit analysis on the Bioanalyzer™ instrument	Agarose gel analysis
100 ng	1 µL	—
1 µg	1 µL of 1:10 dilution	5 µL

Proceed immediately to "End-repair and purify DNA".

End-repair and purify DNA

Materials required

The following materials are provided in the Ion Xpress™ Plus Fragment Library Kit:

- 5X End Repair Buffer
- End Repair Enzyme

Other materials:

- Nuclease-free Water
- 1.5-mL Eppendorf LoBind™ tube
- Agencourt™ AMPure™ XP Reagent
- Magnetic rack

End-repair

Before use, pulse-centrifuge components of the Ion Xpress™ 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** as indicated in the following table:

Input amount	Total volume
100 ng	79 µL
1 µg	158 µL

2. Combine the following components in a 1.5-mL Eppendorf LoBind™ tube, then mix by pipetting.

Component	Volume	
	100-ng input	1-µg input
Fragmented gDNA (from step 1)	79 µL	158 µL
5X End Repair Buffer	20 µL	40 µL
End Repair Enzyme	1 µL	2 µL
Total volume	**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 (1.8X sample volume) to the sample, then pipet up and down 5 times to mix the bead suspension with the DNA thoroughly. Pulse-centrifuge, then incubate at room temperature for 5 minutes.

Input amount	Agencourt™ AMPure™ XP Reagent volume
100 ng	180 µL
1 µg	360 µL

2. Pulse-centrifuge, then place the sample tube in a magnetic rack such as the DynaMag™-2 Magnet magnet for 3 minutes or until the solution clears. Remove, then 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 around the tube in the magnet twice to agitate the beads. After the solution clears, remove, then discard the supernatant without disturbing the pellet.

4. Repeat step 3 one more time.

5. To remove residual ethanol, pulse-centrifuge the tube, place it back in the magnetic rack, then 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, then 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-centrifuge, then 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.

Proceed to Chapter 5, “Ligate adapters, nick-repair, and purify the ligated DNA”.

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

Materials required

The following materials are provided in the Ion Xpress™ Plus Fragment Library Kit:

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

The following materials are provided in the Ion Xpress™ Barcode Adapters Kit:

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

Other materials:

- 0.2-mL PCR tubes
- Thermal cycler
- Nuclease-free Water
- Agencourt™ AMPure™ XP Reagent
- Freshly prepared 70% ethanol
- Magnetic rack

Ligate and nick-repair

Use the protocol appropriate for your library.

- For 600-base-read libraries prepared with the Ion Xpress™ Plus Fragment Library Kit, proceed to the “600-base-read libraries sheared using the Ion Xpress™ Plus Fragment Library Kit” on page 42.
- For all other libraries, proceed to the “Standard procedure” on page 44.

600-base-read libraries sheared using the Ion Xpress™ Plus Fragment Library Kit

IMPORTANT!

- 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.

1. Dilute adapters as described in the following table:

   IMPORTANT! If you are using barcoded libraries, be especially careful not to cross-contaminate the barcoded adapters. Change gloves frequently and open one tube at a time.

Component	Volume	
	50–100-ng DNA input	1-µg DNA input
Non-barcoded libraries		
Adapters [1]	1 µL	3 µL
Nuclease-free Water	3 µL	9 µL
Total volume	**4 µL**	**12 µL**
Barcoded libraries[2]		
Ion P1 Adapter	1 µL	3 µL
Ion Xpress™ Barcode X [3]	1 µL	3 µL
Nuclease-free Water	2 µL	6 µL
Total volume	**4 µL**	**12 µL**

[1] Ion Plus Fragment Library Kit Adapters (green cap)
[2] Use a separate tube for each barcode.
[3] X = chosen barcode

2. In a new 0.2-mL PCR tube, combine the following reagents, then mix well by pipetting up and down.

Component	Volume	
	50–100-ng DNA input	1-µg DNA input
DNA	~25 µL	~25 µL
10X Ligase Buffer	10 µL	10 µL
Adapters mix from step 1	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 volume	**100 µL**	**100 µL**

3. Place the tube in a thermal cycler, then run the following program.

Stage	Temperature	Time
Hold	25°C	15 minutes
Hold	72°C	5 minutes
Hold	4°C	Hold[1]

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

4. Transfer the entire reaction mixture to a new 1.5-mL Eppendorf LoBind™ tube.

Proceed to “Purify the adapter-ligated and nick-repaired DNA” on page 45.

Standard procedure

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

IMPORTANT!

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

Component	Volume	
	50-100-ng gDNA input	1-µg gDNA input
Reaction setup for non-barcoded libraries		
DNA	~25 µL	~25 µL
10X Ligase Buffer	10 µL	10 µL
Adapters[1]	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 volume	**100 µL**	**100 µL**
Reaction setup for barcoded libraries [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 volume	**100 µL**	**100 µL**

[1] Ion Plus Fragment Library Kit Adapters (green cap)

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

[3] X = chosen barcode

2. Place the tube in a thermal cycler, then run the following program:

Stage	Temperature	Time
Hold	25°C	15 minutes
Hold	72°C	5 minutes
Hold	4°C	Hold[1]

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

3. Transfer the entire reaction mixture to a new 1.5-mL Eppendorf LoBind™ tube.

Proceed to "Purify the adapter-ligated and nick-repaired DNA".

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, vortex thoroughly to mix the bead suspension with the DNA, then pulse-centrifuge the tube. Incubate the mixture for 5 minutes at room temperature.

Library size	Volume of Agencourt™ AMPure™ XP Reagent
400–600-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-centrifuge, then place the tube in a magnetic rack such as the DynaMag™-2 Magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.

3. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol. Incubate for 30 seconds, turning the tube 3-4 times in the magnet to agitate the beads. After the solution clears, remove, then discard the supernatant without disturbing the pellet.

4. Repeat step 3 for a second wash.

5. To remove residual ethanol, pulse-centrifuge the tube, place it back in the magnetic rack. 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, then 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-centrifuge, then place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind™ tube without disturbing the pellet.

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

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

Proceed to size select the unamplified library using one of the following methods:

- Chapter 6, “Size-select the library with the E-Gel™ SizeSelect™ II Agarose Gel”
- Chapter 7, “Size-select the library with the Pippin Prep™ System”

6 Size-select the library with the E-Gel™ SizeSelect™ II Agarose Gel

Start with unamplified library, non-barcoded or barcoded, prepared and purified as described in Chapter 5, “Ligate adapters, nick-repair, and purify the ligated DNA”.

Sequencing system	Library size	Target peak size
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System	600-base-read	~680 bp
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System Ion PGM™ System	500-base-read	~580 bp
	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

To learn more about other size-selection methods, visit the Thermo Fisher Scientific website (**www.thermofisher.com**).

Materials required

- E-Gel™ SizeSelect™ II Agarose Gel, 2% (Cat. No. G661012)
- 10X Sample Loading buffer (included with E-Gel™ SizeSelect™ II Agarose Gels)
- E-Gel™ Sizing DNA Ladder (Cat. No. 10488100)
- E-Gel™ Power Snap Electrophoresis Device (Cat. No. G8100)
- *(Optional)* E-Gel™ Power Snap Camera (Cat. No. G8200)
- Safe Imager™ Viewing Glasses (Cat. No. S37103; included with E-Gel™ Power Snap Electrophoresis Device (Cat. No. G8100))
- Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5-mL (Fisher Scientific™ Cat. No. 13-698-791, Eppendorf™ Cat. No. 022431021
- UltraPure™ DNase/RNase-Free Distilled Water (Cat. No. 10977015, 10977023)

Procedural guidelines

- Use the indicated amount of DNA per well for single or multiple bands:
  - Do not exceed 500 ng of total DNA per one sample lane.
  - For sheared DNA, do not exceed 1 µg.
- For 500-base-read libraries, follow the recommendations for 600-base-read libraries.
- Samples that contain ≥50-mM NaCl, 100-mM KCl, 10-mM acetate ions, or 10 mM EDTA (for example, certain restriction enzymes and PCR buffers) can cause loss of resolution on E-Gel™ agarose gels. For best results, dilute samples that contain high salt levels 2- to 5-fold in low TE.
- Load the E-Gel™ SizeSelect™ II Agarose Gel within 15 minutes of opening the pouch. Run the gel within 1 minute of loading the samples.
- Always wear Safe Imager™ Viewing Glasses when working with the E-Gel™ Power Snap Electrophoresis Device with the cover open.

For more information about using the E-Gel™ SizeSelect™ II Agarose Gel and the E-Gel™ Power Snap Electrophoresis Device, see the *E-Gel™ SizeSelect™ II Agarose Gel Quick Reference* (Pub. No. MAN0017341) and the *E-Gel™ Power Snap Electrophoresis System User Guide* (Pub. No. MAN0017050) found at **thermofisher.com**.

Note: E-Gel™ SizeSelect™ II Agarose Gel is also compatible with the E-Gel™ iBase™ and E-Gel™ Safe Imager Real-Time Transilluminator. The E-Gel™ iBase™ and E-Gel™ Safe Imager Real-Time Transilluminator have been discontinued, but are still supported. For more information, contact Technical Support.

Prepare samples

1. *(1-µg input samples only)* Add 20 µL of Low TE to the purified ligated DNA to bring the total volume to 40 µL, then split the sample into two 20-µL aliquots.
2. Prepare up to 25 µL of sample in 1X Sample Loading Buffer.

IMPORTANT! This step is required, do not omit.

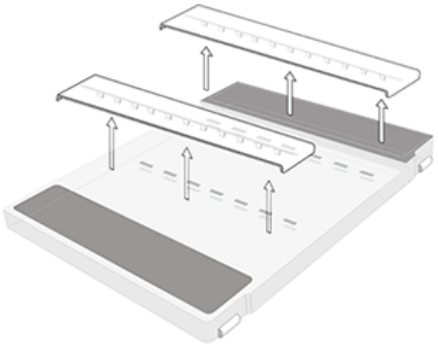
Component	Volume
10X Sample Loading Buffer	2.5 µL
Sample	20 µL
UltraPure™ DNase/RNase-Free Distilled Water	up to 25 µL

Note: You can divide samples with higher amounts of DNA across multiple wells.

Prepare the gel

1. Remove the gel from the packaging.
2. Gently remove the combs from the gel.

IMPORTANT! Do not allow the combs to bend or create suction in the wells during removal.



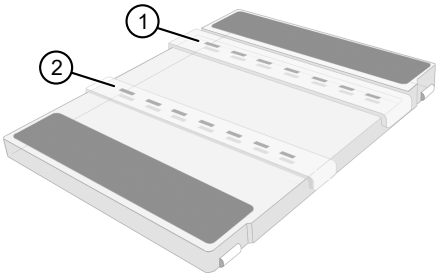
3. Insert the gel cassette into the E-Gel™ Power Snap Electrophoresis Device, starting from the right edge.

Note: If you are using any other electrophoresis device, contact Technical Support.

4. Press down on the left side of the cassette to secure it into the device.

Load samples

1. Fill all sample and recovery wells with 50 µL of deionized water.

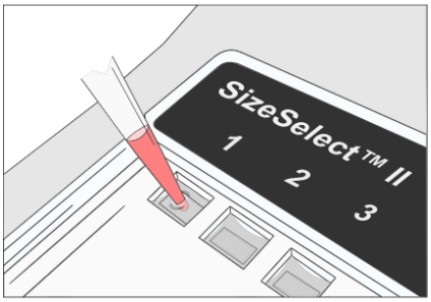


① Sample wells
② Recovery wells

2. Load 25 µL of each sample in 1X Sample Loading Buffer into the sample wells from the bottom up.

Note: Reserve one sample well (marker well) for the ladder.

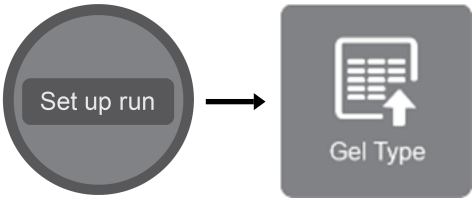
IMPORTANT! Do not pierce the gel or introduce bubbles into the wells.



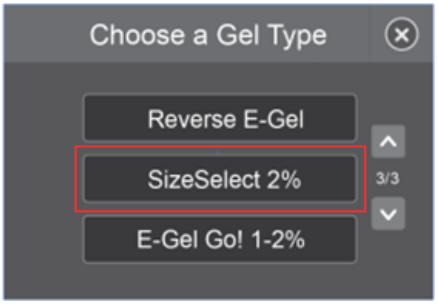
3. Load 25 µL of ready-to-use E-Gel™ Sizing DNA Ladder into the marker well.

Run the gel

1. On the E-Gel™ Power Snap Electrophoresis Device touchscreen, tap **Set up run**, then tap **Gel Type**.



2. In the **Choose a Gel Type** dialog box, tap **SizeSelect 2%**.

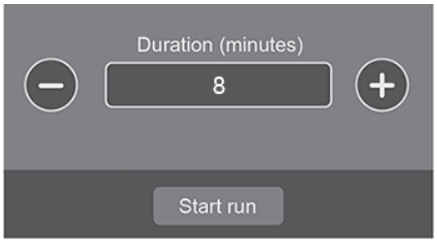


3. Determine the estimated DNA run time. For more information, see “Guidelines for estimating run time” on page 53 .

4. Use the ⊖ and ⊕ buttons to adjust the protocol time according to the expected migration time of the target fragment to the reference line.

5. Tap **Start run** to run the gel protocol.

   Note: The run stops automatically after the programmed time has elapsed.



Check the gel status

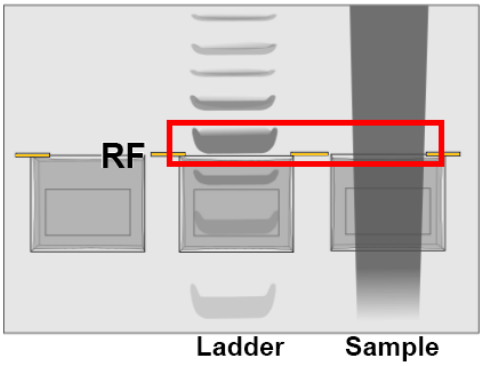
You can monitor the gel during the run to avoid the target fragment passing the recovery well.

Note: For better visibility, reduce the ambient light or work in a dark room.

1. On the instrument touchscreen, tap **Back light** to activate the blue light transilluminator and monitor the gel status during the run.

   Note: The transilluminator turns off automatically after 1 minute.

2. When the reference band of the DNA ladder reaches the reference line (RF) near the row of recovery wells, tap **Pause run (⏸)** to pause the gel.



Prepare the recovery wells

CAUTION! You must put on the Safe Imager™ Viewing Glasses before proceeding with the protocol.

1. Open the filter lid of the E-Gel™ Power Snap Electrophoresis Device, then tap **Back light** on the instrument touchscreen to activate the blue light transilluminator.

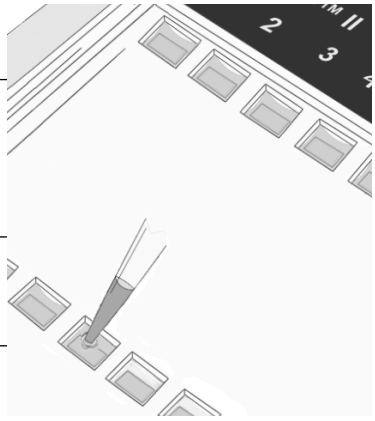
   Note: The transilluminator turns off automatically when the filter lid is opened. Tap **Back light** to re-activate the blue light transilluminator.

2. Carefully remove all liquid from each recovery well.

   IMPORTANT! Do not skip this step. Not removing all the liquid from the recovery wells can result in broad range libraries.

3. Load 25 µL of nuclease-free water into each recovery well.

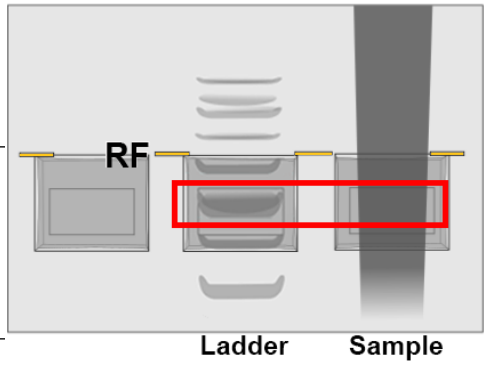
   IMPORTANT! Do not allow water to spill over the edge of the wells.



Collect the DNA fragment

1. Tap **Resume (▶)** to resume the run, then carefully observe as the reference band enters the recovery well.

   IMPORTANT! To determine when to collect samples of specific target library length, see Table 4 in "Guidelines for estimating run time" on page 53.



2. Tap **Done** to end the protocol and stop the gel, then recover the sample with a pipette into a clean 1.5-mL Eppendorf LoBind™ tube.

   IMPORTANT! Avoid piercing the agarose during collection.

   Note:
   - Ensure that you recover the entire volume of the recovery well (~25 µL).
   - Some residual DNA remains visible in the well due to migration into the agarose at the bottom of the well.

3. *(1-µg input samples only)* If you split the sample between two wells, combine the samples collected from the corresponding recovery wells into a single tube.

Proceed to Chapter 8, "Amplify and purify the library". If you are using the Ion Library Equalizer™ Kit (for up to 300-base-read libraries only), proceed to Appendix A, "Equalize the library (for up to 300-base-read libraries)".

Guidelines for estimating run time

- To estimate target DNA run time to the reference line, see Table 3.
- The E-Gel™ Sizing DNA Ladder is also used as a size reference marker. To estimate run time from the reference line to the collection well, see Table 4.
- The run times that are indicated in the tables are estimates. Monitor your gel in real-time during the run to ensure that the sample does not pass the recovery well.
- Identically sized bands in different wells can migrate differently.
- DNA fragment size, amount, and salt content can affect migration rates.

Table 3 E-Gel™ Sizing DNA Ladder migration pattern in 2% E-Gel™ SizeSelect™ II Agarose Gel

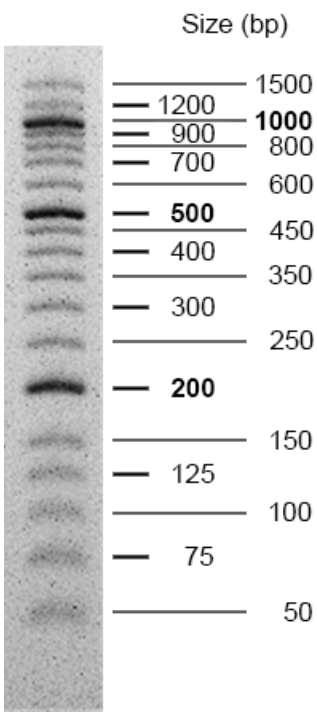
Ladder	Fragment size	DNA amount (per 25 µL)	Estimated migration time to reference line
	1,500 bp	1.5 ng	~ 19.5 minutes
	1,200 bp	1.5 ng	~ 18.5 minutes
	1,000 bp	6 ng	~ 17.5 minutes
	900 bp	2 ng	~ 17 minutes
	800 bp	2 ng	~ 16.5 minutes
	700 bp	2 ng	~ 16 minutes
	600 bp	2 ng	~ 15.5 minutes
	500 bp	6 ng	~ 14.5 minutes
	450 bp	2 ng	~ 14 minutes
	400 bp	2 ng	~ 13.5 minutes
	350 bp	2 ng	~ 13 minutes
	300 bp	2 ng	~ 12.5 minutes
	250 bp	2 ng	~ 11.5 minutes
	200 bp	6 ng	~ 11 minutes
	150 bp	2 ng	~ 10 minutes
	125 bp	2 ng	~ 9.5 minutes
	100 bp	2 ng	~ 9 minutes
	75 bp	2 ng	~ 8.5 minutes
	50 bp	2.5 ng	~ 8 minutes

Table 4 NGS library size selection reference

Library size	Target library peak	Estimated run time to reference line	Input sample amount	Stop the run and collect your sample when...	Band schematic
Ion S5™ System, Ion S5™ XL System, or Ion GeneStudio™ S5 Series System					
600-base-read	680 bp	Varies[1]	50–100 ng, 500 ng	Varies[1]	
Ion PGM™ System, Ion S5™ System, Ion S5™ XL System, or Ion GeneStudio™ S5 Series System					
500-base-read	580 bp	Varies[1]	50–100 ng, 500 ng	Varies[1]	
400-base-read	480 bp	14–20 minutes	500 ng	500 bp band is at the **top** of the **exposed agarose area**	
			50–100 ng	500 bp band **just entered the top edge** of the **recovery well**	
300-base-read	390 bp	13–16 minutes	500 ng	400 bp band is at the **middle** of the **exposed agarose area**	
			50–100 ng	500 bp band is at the **top** of the **exposed agarose area**	
200-base-read	330 bp	12–14 minutes	500 ng	350 bp band is at the **top** of the **exposed agarose area**	
			50–100 ng	350 bp band has **just completely entered the top edge** of the **recovery well**	
100-base-read	200 bp	11–12.5 minutes	500 ng	200 bp band is in the **middle** of the **recovery well**	
			50–100 ng	200 bp band is in the **middle** of the **recovery well**	
Ion Proton™ System					
200-base-read	170 bp	12–14 minutes	500 ng	300 bp band is at the **top** of the **exposed agarose area**	
			50–100 ng	300 bp band is in the **middle** of the **exposed agarose area**	
150-base-read	220 bp	11–14.5 minutes	500 ng	200 bp band is in the **middle** of the **exposed agarose area**	

Table 4 NGS library size selection reference *(continued)*

Library size	Target library peak	Estimated run time to reference line	Input sample amount	Stop the run and collect your sample when...	Band schematic
Ion S5™ System, Ion S5™ XL System, or Ion GeneStudio™ S5 Series System					
150-base-read	220 bp	11–14.5 minutes	50–100 ng	200 bp band is in the **middle** of the **exposed agarose area**	

[1] Monitor the gel during the run to avoid the target fragment passing the recovery well. Use the E-Gel™ Sizing DNA Ladder to estimate the migration time and the location of your library fragment.

Troubleshooting for E-Gel™ size selection

Observation	Possible cause	Recommended action
Low yield	Loading volume was incorrect.	Load up to 25 µL of prepared sample per well.
	Recovery wells were not pre-filled with deionized water prior to elution.	Once target fragment reaches reference line, pause the run and fill all recovery wells with deionized water.
	Target DNA did not completely enter the gel.	Increase the DNA fragment collection time.
	Target DNA passed the recovery lane.	Carefully observe the DNA migration into the recovery well. Minimize ambient light or perform the workflow in dark room.
		Run the Reverse E-gel protocol to move the target DNA band back into the recovery well (see *E-Gel™ Power Snap Electrophoresis System User Guide* (Pub. No. MAN0017050)).
	DNA amount was too high.	Collect DNA from the well in two or more fractions.
		Make sure to load the recommended DNA amount.
Poor resolution or smearing of bands	The sample was overloaded.	Do not exceed 500 ng of total DNA per one sample lane or 500 ng of DNA per one band. For sheared DNA, do not exceed 1 µg.
	Salt concentration in samples was too high.	Dilute your samples 2– to 5–fold in low TE or nuclease-free water.
	Total sample volume was too low or too high.	Use the recommended volume of 25 µL per sample lane.
	Loading wells were not pre–filled with deionized water before loading the sample.	Fill all gel wells with 50 µL of deionized water before loading any sample or a ladder.
	The sample was not prepared properly.	Prepare up to 25 µL of sample in 1X concentration of 10X Sample Loading Buffer.

Observation	Possible cause	Recommended action
Cannot detect target DNA band	Ambient light was high, or sample DNA concentration was too low.	Perform the workflow in dark room environment to minimize ambient light.
Target DNA band passed the recovery well	Selected protocol time was too long.	Run the Reverse E-gel protocol to move the target DNA band back into the recovery well (see *E-Gel™ Power Snap Electrophoresis System User Guide* (Pub. No. MAN0017050)).
DNA migration exhibits smiley effect	The gel run time was too long.	Do not run gels longer for longer than 30 minutes.
	The gel that was used was expired.	Use a fresh gel for size selection procedure.
	Loading conditions were incorrect.	Follow the sample loading recommendations.

7 Size-select the library with the Pippin Prep™ System

Start with unamplified library, non-barcoded or barcoded, prepared and purified as described in Chapter 5, "Ligate adapters, nick-repair, and purify the ligated DNA".

Sequencing system	Library size	Target peak size
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System	600-base-read	~680 bp
	500-base-read	~570 bp
	400-base-read	~480 bp
	300-base-read	~390 bp
	200-base-read	~330 bp
	100-base-read	~200 bp
Ion PGM™ System	500-base-read	~570 bp
	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 Thermo Fisher Scientific website (**www.thermofisher.com**) for other library size-selection methods.

IMPORTANT! The protocol in this chapter 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 versions 5.8 and later are required to run 2% gels with Marker L.

Materials required

- Low TE (from Ion Plus Fragment Library Kit)
- Pippin Prep™ System (Sage Science, Cat. No. PIP0001)
- Pippin Prep™ Kit 2010: includes 2% Agarose Gel Cassettes (ethidium), Loading Solution, Marker B, and Electrophoresis Buffer (Sage Science, Cat. No. CSD2010)
- Pippin Prep™ Kit CDF 2010: includes 2% Agarose Gel Cassettes (dye free), Loading Solution/Marker L mix, and Electrophoresis Buffer (Sage Science, Cat. No. CDF2010)
- Pippin Prep™ Kit CDF 1510-FAST: includes 1.5% Agarose Gel Cassettes (dye free), internal standard K, and Electrophoresis Buffer (Sage Science, Cat. No. CDF1510)
- Nuclease-free Water
- Agencourt™ AMPure™ XP Kit
- Freshly prepared 70% ethanol
- Magnetic rack

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

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

For 100–300-base-read libraries

1. From the cassette type dropdown list , select **2% Marker B No Overflow Detection**.
2. Select the **Tight** collection mode for each lane, then define the **BP Target** setting for each of 1–4 lanes used.

Sequencing system	Library size	BP Target setting
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System 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, then selecting the **Apply Reference to all Lanes** button. Ensure that the **Ref Lane** value for each lane is 5.
4. Set the run time to 1.5 hours.

For 400- and 500-base-read libraries

1. From the cassette type dropdown list, select **2% DF Marker L**.
2. Select the **Tight** collection mode for each lane, then define the **BP Target** setting for each of 1–5 lanes used.

Sequencing system	Library size	BP Target setting
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System Ion PGM™ System	400-base-read	475 bp
	500-base-read	570 bp

3. Define lanes 1–5 as sample lanes, then press the **Use Internal Standards** button to match the lane numbers, then ensure that the **Ref Lane** values match the lane numbers.
4. Set the run time to 1.5 hours.

For 600-base-read libraries

1. From the cassette type dropdown list, select **1.5% DF Marker K**.
2. Select the **Tight** collection mode for each lane, then define the **BP Target** setting for each lane as 685 bp.

   Note: Sometimes, the Pippin Prep™ instrument can under- or over-select this target. Adjust your **BP Target** setting based on your laboratory conditions.
3. Define lanes 1–5 as sample lanes, press the **Use Internal Standards** button to match the lane numbers, then ensure that the **Ref Lane** values match the lane numbers.
4. Set the run time to 1.5 hours.

Prepare the 1.5% or 2% Agarose Gel cassette

Note: For 600–base–read libraries use the 1.5% Agarose Gel cassettes. For 500–base–read libraries and smaller use the 2% Agarose Gel cassettes.

1. Unwrap the 1.5% or 2% Agarose Gel cassettes, then tip the cassette toward the loading wells end to dislodge any air bubbles present around the elution wells. Insert the cassette into the instrument. Tap the bottom of the cassette until there are no bubbles left behind the elution wells.
2. Insert the cassette into the instrument.
3. Remove the two adhesive strips covering the loading wells and elution wells.
4. Fill the loading wells with Electrophoresis Buffer to the top so that a concave meniscus forms.
5. Remove all liquid from the elution wells, then add 40 µL of Electrophoresis Buffer.
6. Seal the elution wells with the adhesive tape strips supplied with the cassette packaging.
7. Following the instructions in the Pippin Prep™ System 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 select the newly generated separation file (or a previously saved file) from the **Protocol Name** dropdown list.
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 400–500-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 select the newly generated separation file (or a previously saved file) from the **Protocol Name** dropdown list.
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.

For 600-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 Marker K. 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 pipettor 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 this procedure.

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

1. Add Agencourt™ AMPure™ XP Reagent.

Library	Agencourt™ AMPure™ XP Reagent
600-base-read	42 µL (0.7X sample volume)
500-base-read or smaller	90 µL (1.5X sample volume)

2. Vortex to thoroughly mix the bead suspension with the DNA, pulse-centrifuge the tube, then incubate the mixture for 5 minutes at room temperature.
3. Pulse-centrifuge, then place the tube in a magnetic rack such as the DynaMag™-2 Magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.
4. Leaving the tube on the magnet, add 500 µL of freshly prepared 70% ethanol to the sample. Incubate for 30 seconds, turning the tube 3-4 times in the magnet to agitate the beads. After the solution clears, remove, then discard the supernatant without disturbing the pellet.
5. Repeat step 4 for a second wash.
6. To remove residual ethanol, centrifuge the tube, place it back in the magnetic rack, then 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.

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

8. 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 82. Otherwise, proceed to step 9.

9. Remove the tube from the magnetic rack, then add the indicated volume of Low TE directly to the pellet to disperse the beads.

Input amount	Low TE volume
50–100 ng	25 µL
1 µg	50 µL

10. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds to mix thoroughly.

11. Pulse-centrifuge, then place the tube in the magnetic rack for at least 1 minute or 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.

Proceed to Chapter 8, "Amplify and purify the library".

8 Amplify and purify the library

Determine if 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 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.

Note: 600-base-read libraries 50–100-ng input: proceed to "Amplify the library" on page 68.

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

Library normalization/quantification options[1] (40–70 minutes)			
Method	Ion Library Equalizer™ Kit	qPCR [2]	Bioanalyzer™ instrument analysis
Features	No library quantification required	Amplification optional[1]	Amplification optional[1]
Time	~40 minutes	~70 minutes	~60 minutes

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

[2] Quantify the unamplified library by qPCR with the Ion Library TaqMan™ Quantitation Kit (Cat. No. 4468802). This kit directly determines library concentration so that the library can be appropriately diluted for template preparation.

Procedure for 100–500-base-read libraries

1. Determine the concentration of the unamplified library with the Ion Library TaqMan™ Quantitation Kit. Follow the instructions in the *Ion Library TaqMan™ Quantitation Kit User Guide* (Pub. No. MAN0015802), then 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 using the following equation:

   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.

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

Library amplification	Proceed to
Yes	"Amplify the library" on page 68
No	• Chapter 9, "Qualify non-barcoded libraries", or • Chapter 10, "Qualify and pool barcoded libraries"

Procedure for 600-base-read libraries (1-µg input)

1. **Note:** Quantify the library using the Qubit™ Fluorometer with the Qubit™ dsDNA HS Assay Kit. Follow the instructions in the *Qubit™ dsDNA HS Assay Kits User Guide* (MAN0002326).

   Determine the amplified library concentration:
   a. Make a 1:200 working dilution of Qubit™ dsDNA HS Reagent using the Qubit™ dsDNA HS Buffer.
   b. Combine 10 µL of the amplified Ion AmpliSeq™ library with 190 µL of dye reagent, mix well, then incubate for at least 2 minutes.
   c. Prepare each Qubit™ standard as directed in the user guide.
   d. Measure the concentration using the Qubit™ Fluorometer.
   e. Calculate the concentration of the undiluted library by multiplying by 20.

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

Library insert size	Concentration in ng/mL (~100 pM)
100 bp	12
200 bp	18
300 bp	25
400 bp	32
500 bp	38
600 bp	45

   For example, with a barcoded 600-bp library (~680 bp with barcoded adapters):
   - The library concentration is 270 ng/mL.
   - The dilution factor is 270 ng/mL divided by 45 ng/mL = 6
   - Therefore, 10 µL of library that is mixed with 50 µL of Low TE (1:6 dilution) yields approximately 45 ng/µL (~100 pM).

3. Dilute library to ~100 pM as described, then proceed to template preparation, or combine or store libraries at 4–8°C for up to 1 month. For longer term, store at –30 to –20°C.

Amplify the library

Materials required

The following materials are provided in the Ion Plus Fragment Library Kit:

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

Other materials:

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

Amplify the library

1. Adjust the volume of the unamplified library as described in the following table.

Size-selection method	Library input amount	Volume to add to the amplification reaction in step 2
E-Gel™ SizeSelect™ II Agarose Gel *or* Pippin Prep™ System	50–100 ng	25 µL
	1 µg	50 µL

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

IMPORTANT!

- 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.

Component	Volume	
	50–100-ng DNA input	1-µg DNA input
Platinum™ PCR SuperMix High Fidelity	100 µL	200 µL
Library Amplification Primer Mix	5 µL	10 µL
Unamplified library	25 µL	50 µL
Total volume	**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, then run the PCR cycling program as described in Table 5. Set the number of cycles according to Table 6.

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

Table 5 PCR cycling conditions

Stage	Step	Temperature	Time
100–500-base-read libraries			
Holding	Denature	95°C	5 minutes
Cycling[1]	Denature	95°C	15 seconds
	Anneal	58°C	15 seconds
	Extend	70°C	1 minute
Holding	—	4°C	Hold[2]
600-base-read libraries			
Holding	Denature	95°C	5 minutes
Cycling[1]	Denature	95°C	15 seconds
	Anneal	58°C	15 seconds
	Extend	70°C	1.5 minutes
Holding	Extend	70°C	5 minutes
Holding	—	4°C	Hold[2]

[1] Set the number of cycles according to Table 6.
[2] Not a stopping point; continue directly to the next steps.

Table 6 Recommended number of cycles

Library input	Number of cycles
50–100 ng	8
1 µg	5

5. Combine previously split PCR reactions 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 this procedure.

1. Add the indicated volume of Agencourt™ AMPure™ XP Reagent to each sample:

Input	Volume of Agencourt™ AMPure™ XP Reagent			
	100–150-base-read library (1.5X sample volume)	200–300-base-read library (1.2X sample volume)	400–500-base-read library (1X sample volume)	600-base-read library (0.7X sample volume)
50–100 ng	195 µL	156 µL	130 µL	91 µL
1 µg	390 µL	312 µL	260 µL	182 µL

2. Vortex to mix the bead suspension thoroughly with the DNA. Pulse-centrifuge, then incubate the mixture for 5 minutes at room temperature.

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

4. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol. Incubate for 30 seconds, turning the tube around 3-4 times in the magnet to agitate the beads. After the solution clears, remove, then discard the supernatant without disturbing the pellet.

5. Repeat step 4 for a second wash.

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

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

8. Remove the tube from the magnetic rack, then add 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-centrifuge, then place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind™ tube without disturbing the pellet.

   IMPORTANT! The supernatant contains the 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, then 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.

Proceed to qualify the libraries.

- **For non-barcoded libraries**, proceed to Chapter 9, “Qualify non-barcoded libraries”.
- **For barcoded libraries**, proceed to Chapter 10, “Qualify and pool barcoded libraries”.

9 Qualify non-barcoded libraries

For barcoded libraries, go to Chapter 10, “Qualify and pool barcoded libraries”.

Evaluate the size distribution of the library

Analyze an aliquot of the library on the Agilent™ 2100 Bioanalyzer™ instrument using the Agilent™ High Sensitivity DNA Kit, as indicated in the following table.

See Figures 7–15 in Appendix D, “Example Bioanalyzer™ traces” for example traces.

Input amount	Library aliquot volume and dilution factor	
	Unamplified library	Amplified library
50–100 ng	N/A	1 µL of 1:10 dilution
100	1 µL	
1 µg	1 µL of 1:5 dilution	

IMPORTANT! Ensure that excessive amounts of primer-dimers (immediately adjacent to the lower marker) or over-amplification products (concatamers) 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 appropriate for template preparation.

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

Amplified libraries: Determine the library dilution using Agilent™ 2100 Bioanalyzer™ instrument analysis *or* by qPCR.

Quantification method	Features
Ion Library TaqMan™ Quantitation Kit (qPCR)	• Quantitative real-time PCR (qPCR) methodology. • Direct determination of the library concentration from a standard curve. • Higher precision for quantitation. A single dilution of the library is usually sufficient for an optimized template preparation procedure. • Higher sensitivity for detection. The Ion Library TaqMan™ Quantitation Kit is recommended for unamplified or low-yield libraries. Libraries with insufficient material for detection by 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 TaqMan™ Quantitation Kit accurately quantifies the properly adapted libraries with minimal impact from background material.
Bioanalyzer™ 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 TaqMan™ Quantitation Kit to determine the library dilution.
- Use Agilent™ 2100 Bioanalyzer™ instrument analysis to evaluate the size distribution of the library.

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

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

2. Use the following equation to determine the dilution factor that results in a concentration of ~100 pM:

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

   Note:
   - 100 pM concentration is optimal for downstream template preparation.
   - 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 (see “Determine if amplification is required” on page 65).

   Example:

   The library concentration is 15,000 pM.

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

   Therefore, 1 µL of library that is mixed with 149 µL of Low TE (1:150 dilution) yields approximately 100 pM. Use this library dilution for template preparation.

For quantifying libraries for use in the Ion PGM™ Template IA reaction, see the *Ion PGM™ Template IA 500 Kit User Guide* (Pub. No. MAN0009347).

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

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

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

   Note: For quantifying libraries for use in the Ion PGM™ Template IA reaction, see the *Ion PGM™ Template IA 500 Kit User Guide* (Pub. No. MAN0009347).

2. Use the following equation to determine the dilution factor that results in a concentration of ~100 pM:

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

   Note: 100-pM concentration is optimal for downstream template preparation.

   Example:

   The library concentration is 15,000 pM.

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

Therefore, 1 µL of library that is 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

Before 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 Torrent™-branded template preparation kit.

10 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 quantification method.

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

For non-barcoded libraries, follow Chapter 9, “Qualify non-barcoded libraries”.

Evaluate 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.

For example traces, see Figures 7–15 in Appendix D, “Example Bioanalyzer™ traces”.

Input amount	Library aliquot volume and dilution factor	
	Unamplified library	Amplified library
50–100 ng	N/A	1 µL of 1:10 dilution
100 ng	1 µL	
1 µg	1 µL of 1:5 dilution	

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

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

1. Use the Ion Library TaqMan™ 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 TaqMan™ Quantitation Kit User Guide* (Cat. No. MAN0015802).

   IMPORTANT! Fast cycling can result in inaccurate quantification when quantifying libraries ≥300-bp. We recommend using standard qPCR cycling conditions that are specified for ≥300-bp libraries. For more information, see the *Ion Library TaqMan™ Quantitation Kit User Guide* (Cat. No. MAN0015802).

2. Determine the dilution factor that gives a concentration of ~100 pM. This concentration is appropriate 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

   Therefore, 1 µL of library pool that is 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 (see “Determine if amplification is required” on page 65). 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 is at the correct concentration for template preparation.

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

1. From the analysis used to evaluate the individual barcoded library size distribution, determine the molar concentration in pmol/L of each barcoded library using the Bioanalyzer™ software. If needed, 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 that is calculated for your library pooling algorithm.
   - Alternatively, confirm the concentration of the library pool by analyzing 1 µL of the library pool on the Agilent™ 2100 Bioanalyzer™ instrument using the Agilent™ High Sensitivity DNA Kit, then determine the molar concentration of the library pool using the Bioanalyzer™ software. If needed, 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 appropriate 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

   Therefore, 1 µL of library pool that is 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

Before 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 Torrent™-branded template preparation kit.

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

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. To equalize the library, amplify, then capture the library on the Equalizer™ Beads. After elution of the equalized library, proceed directly to combining libraries or template preparation.

IMPORTANT! E-Gel™ SizeSelect™ II Agarose Gel size-selected libraries require purification before equalization.

Materials required

The following materials are provided in the Ion Plus Fragment Library Kit:

- Platinum™ PCR SuperMix High Fidelity

The following materials are provided in the Ion Library Equalizer™ Kit:

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

The following materials are required for E-Gel™ SizeSelect™ II Agarose Gel size-selected libraries only:

- Agencourt™ AMPure™ XP Reagent (Beckman Coulter™ Cat. No. A63880)
- 70% Ethanol (freshly prepared)

Other materials:

- 0.2-mL PCR tubes
- Low TE
- Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5-mL
- Magnetic rack
- Thermal cycler

Purify the library *(E-Gel™ SizeSelect™ II Agarose Gel size-selected libraries only)*

If you performed library size selection with the E-Gel™ SizeSelect™ II Agarose Gel, purify the library before equalization. If you performed library size selection with the Pippin Prep™ System, proceed directly to "Amplify the library" on page 82.

IMPORTANT! Use **freshly prepared 70% ethanol** (1 mL plus overage per sample) for this procedure.

Start warming the Ion Library Equalizer™ Kit reagents to room temperature before proceeding.

1. Add Agencourt™ AMPure™ XP Reagent (1.8X sample volume) to each sample according to the following table.

Sample input amount	Sample volume	Agencourt™ AMPure™ XP Reagent volume
50–100 ng	~30 µL	54 µL
1 µg	~60 µL	108 µL

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

3. Pulse-centrifuge the tube, then place the tube in a magnetic rack for 3 minutes or until the solution is clear.

4. Carefully remove, then discard the supernatant without disturbing the pellet at the bottom of the tube.

5. Leaving the tube on the magnet, add 500 µL of freshly prepared 70% ethanol to each sample. Incubate for 30 seconds, turning the tube twice in the magnet to agitate the beads. After the solution clears, remove, then discard the supernatant without disturbing the pellet.

6. Repeat step 5 for a second wash.

7. To remove residual ethanol, pulse-centrifuge the tube, place the tube back in the magnetic rack, then 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: Evaluate the dryness of the bead pellet by rotating the plate 90 degrees in the magnet. The pellet should migrate slowly. Do not overdry.

Proceed immediately to "Amplify the library" on page 82.

Amplify the library

Before you proceed, ensure that the reagents in the Ion Library Equalizer™ Kit are warmed to room temperature.

1. Remove the tube from the magnet, then add 50 µL of Platinum™ PCR SuperMix High Fidelity (black cap) and 2 µL of Equalizer™ Primers to the bead pellet. The Platinum™ PCR SuperMix High Fidelity and primers can 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.

2. 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.

3. Seal the tube, place it in the thermal cycler, then 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 Table 7.
[2] Not a stopping point; continue directly to the next steps.

Table 7 Recommended number of cycles

Library Input	Number of cycles
50–100 ng	8
1 µg	5

Prepare the Equalizer™ Beads

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

   Note: Beads for multiple reactions can be prepared in bulk.
3. Place the tube in a magnetic rack for 3 minutes or until the solution is 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, then 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 can 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, then 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, then 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, then incubate for 2 minutes or until the solution is clear.
6. Carefully remove and discard the supernatant without disturbing the pellet, then add 150 µL of Equalizer™ Wash Buffer to the reaction.
7. Turn around the tube 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, then add 150 µL of Equalizer™ Wash Buffer to the reaction.
9. Turn around the tube 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 Equalizer™ Wash Buffer as possible is removed without disturbing the pellet.

Elute the equalized library

1. Remove the tube from the magnet, then add 100 µL of Equalizer™ Elution Buffer to the pellet. Pipet up and down 5 times to mix thoroughly.
2. Seal the tube, then incubate in a thermal cycler at 35°C for 5 minutes.
3. Place the tube in the magnet, then 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 in "(Optional) Combine equalized libraries" on page 84.

   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 for your Ion PGM™ System, Ion Proton™ System, Ion S5™ System, Ion S5™ XL System, or Ion GeneStudio™ S5 Series System.

Template preparation documentation is available at **thermofisher.com**.

B

Prepare adapter-compatible DNA: physical fragmentation with the Bioruptor™ Sonication System

This chapter describes conditions for physically shearing gDNA with the Bioruptor™ Sonication Systems. The sonicator generates DNA fragments suitable for preparing 100–500-base-read libraries for use with Ion Torrent™-branded sequencing systems, including the 150- and 200-base-read libraries for the Ion Proton™ System. The sonicated DNA is ready for end-repair. After fragmentation, prepare the libraries by adjusting the downstream size-selection of the library molecules.

Required materials

For ordering information and compatibility, contact the manufacturer.

- Diagenode™ Bioruptor™ Standard Sonication System *or* Bioruptor™ NGS Sonication System
- Bioruptor™ 0.5/0.65-mL tube holder
- Diagenode™ 0.65-mL Bioruptor™ Microtubes
- Low TE (10 mM Tris pH 8.0, 0.1 mM EDTA; from Ion Plus Fragment Library Kit)

Prepare the samples for sonication

Note:

- 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.

Before you proceed, prepare high-quality, RNA-free gDNA. For guidelines, see "Prepare genomic DNA" on page 32.

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 not supplied" on page 15.

2. If running a control, prepare a control sample of 100 ng or 1 µg of control DNA in 50 µL Low TE. Keep the sample on ice. Treat this control as a sample and follow the standard procedure.

   If running a control in addition to samples, prepare the control in a separate tube.

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 sonication:

   - "Bioruptor™ CD-200 TS Sonication System" on page 87
   - "Bioruptor™ UCD-600 NGS Sonication System" on page 89

Bioruptor™ CD-200 TS Sonication System

For 100–300-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): 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 minutes, then sonicate according to the following table:

   Note: Between each cycle, remove 1 cm (150 mL) of the water from the Bioruptor™ tank, then add 250 mL of crushed ice to the fill line.

Sequencing system	Library size	Number of 15-minute cycles	Total sonication time
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System 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

① Power level dial
② Timer (15 minutes maximum)
③ Interval (sonication time–red dial; cool down time–green dial)
④ Rotor with 12 × 0.65-mL tubes
⑤ Water bath

4. Remove the tubes from the rotor, then store on ice.

Proceed to “Assess the fragmentation profile” on page 38.

For 400- and 500-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 minutes, then sonicate.
4. Remove 1 cm (150 mL) of the water from the Bioruptor™ tank, then add 250 mL of crushed ice to the fill line.
5. Set the timer to 9 minutes minutes, then sonicate.

Proceed to “Assess the fragmentation profile” on page 38.

Bioruptor™ UCD-600 NGS Sonication System

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

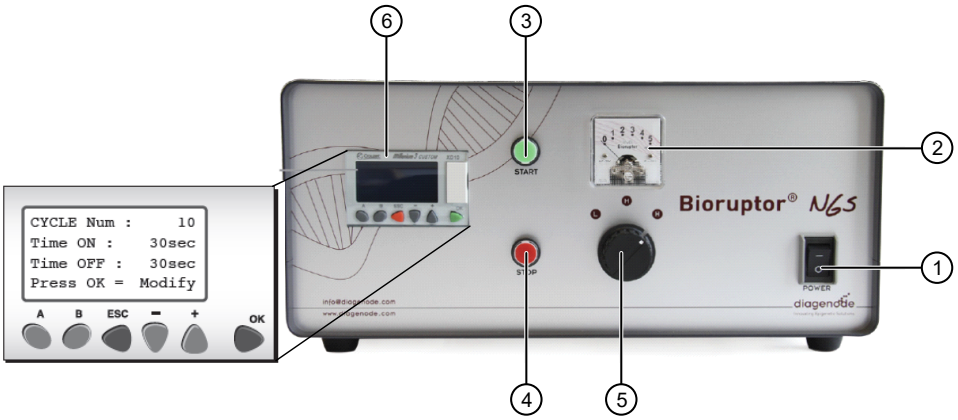
For 100–200-base-read libraries (including the 150- and 200-base-read libraries for the Ion Proton™ System), use the following parameters:

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-, 400-, and 500-base-read libraries, use the following parameters:

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- and 500-base-read)
Power Level	H (High)

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



① Power switch
② Intensity level meter
③ Start button
④ Stop button
⑤ Intensity settings button
⑥ Digital timer

2. Fill the Bioruptor™ Sonication System to just above the fill line with water.
3. Switch on the Bioruptor™ water cooler, then set the temperature to **4°C**.
4. After the set temperature reaches 4°C, insert the rotor containing tubes into the sonicator, then press **START**.

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

   Bioruptor™ Running displays 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- and 500-base-read libraries.
5. Remove the tubes from the rotor, then store on ice.

Proceed to “Assess the fragmentation profile” on page 38.

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.

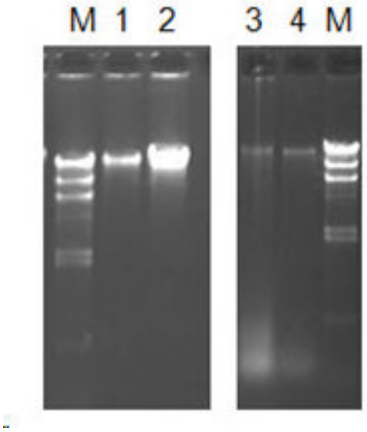


Figure 1 Examples of genomic DNA preparations

High-quality DNA with no contaminating RNA (lanes 1 and 2) is 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-digested molecular weight marker.

If your DNA preparation shows RNA contamination, treat it with RNase I, as described in "(Optional) Treat the DNA with RNase I" on page 92.

(Optional) Treat the DNA with RNase I

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

Materials required:

- RNase I
- PureLink™ columns from the PureLink™ Genomic DNA Mini 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.

Procedure

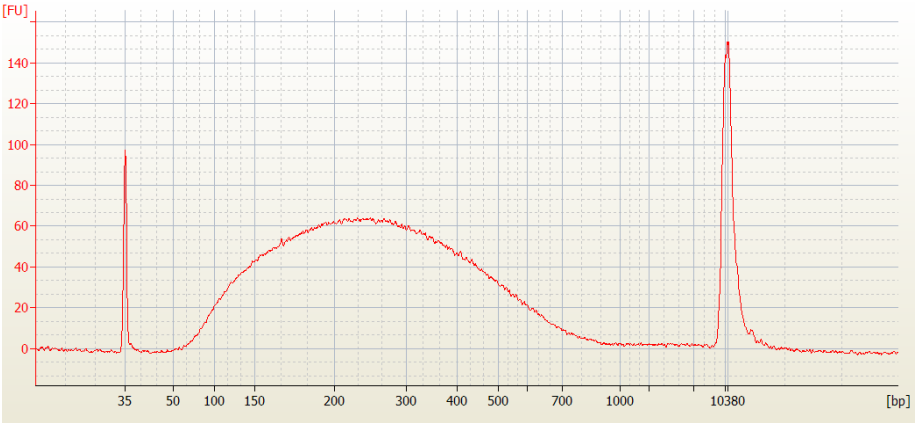
1. Treat the DNA with RNase I according the manufacturer's instructions.
2. Remove the buffer used for RNase I treatment. For example, use a PureLink™ spin column from the PureLink™ Genomic DNA Mini Kit (follow the "Purification Procedure Using Spin Columns" protocol provided in the *PureLink™ Genomic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

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

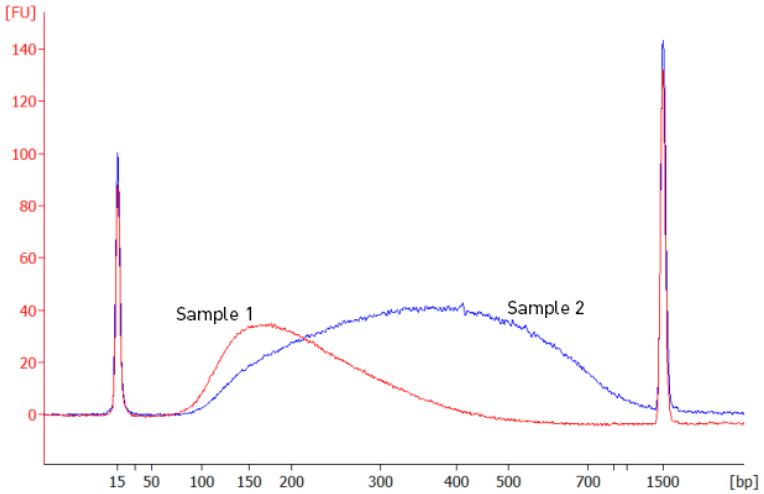
D Example Bioanalyzer™ traces

DNA fragmented with Ion Xpress™ Plus Fragment Library Kit

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



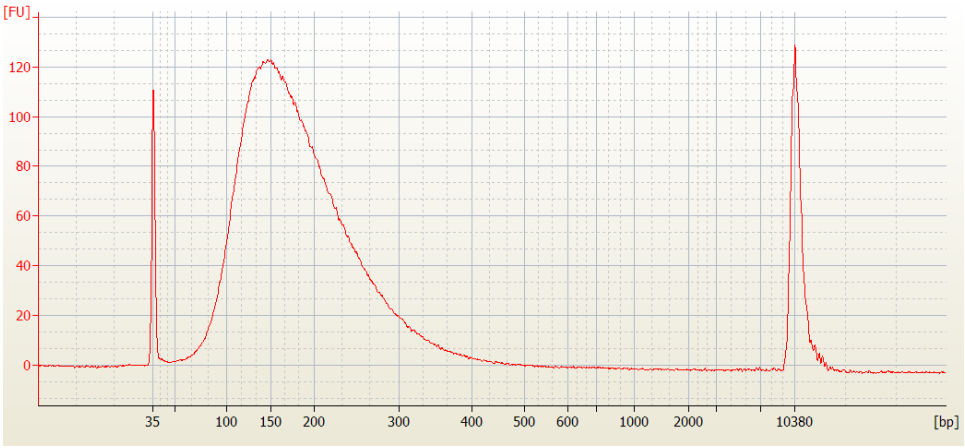
Panel A, Optimal fragmentation profile: 1 µg of *E. coli* DH10B DNA was fragmented with the Ion Xpress™ Plus Fragment Library Kit with a reaction time that was adjusted for 200-base-read libraries. Analysis was performed using the Agilent™ High Sensitivity DNA Kit. Peaks at 35 bp and 10,380 bp represent low- and high-molecular weight markers.



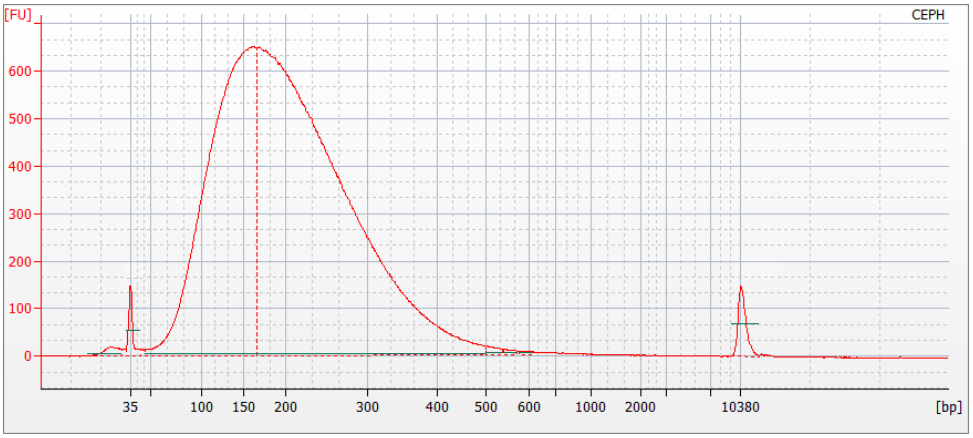
Panel B, Over-, and under-digestion profiles: Examples of over-digested (Sample 1, red) and under-digested (Sample 2, blue) gDNA fragments for a 200-base-read library. Either of these fragmentation profiles can be used for library construction. The expected library yield is greater than library yield from optimally fragmented DNA using either the Bioruptor™ or Covaris™ systems, due to the higher ligation efficiency of fragments generated with Ion Xpress™ Plus Fragment Library Kit.

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

Genomic DNA was fragmented with the Ion Xpress™ Plus Fragment Library Kit with a reaction time that was adjusted for 100-base-read libraries. Analysis was with the Agilent™ High Sensitivity DNA Kit. Peaks at 35 bp and 10,380 bp represent low- and high-molecular weight markers.

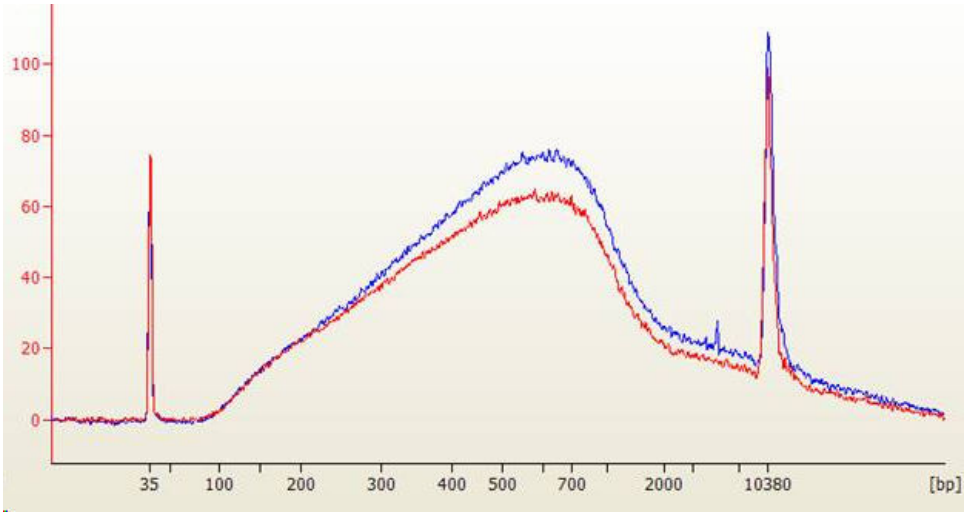


Panel A, Fragment profile of 1 µg of *E. coli* DH10B DNA supplied in the Ion Control Materials 200 Kit (Cat. No. 4471249) for Ion PGM™ System sequencing.



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.

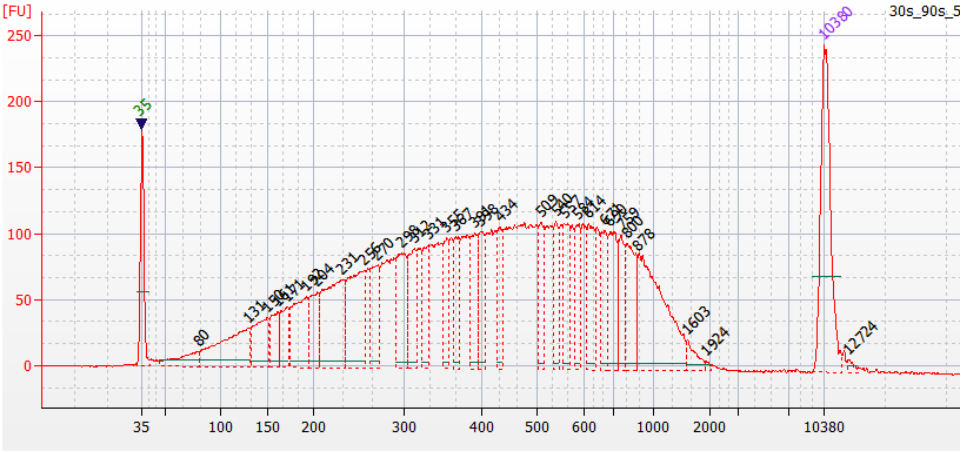
Figure 3 Bioanalyzer™ instrument analysis of genomic DNA fragmented for 500-base-read libraries



1 µg of human gDNA was fragmented with the Ion Xpress™ Plus Fragment Library Kit with a reaction time that was adjusted for 500-base-read libraries. Analysis was with the Agilent™ High Sensitivity DNA Kit. Peaks at 35 bp and 10,380 bp represent low- and high-molecular weight markers.

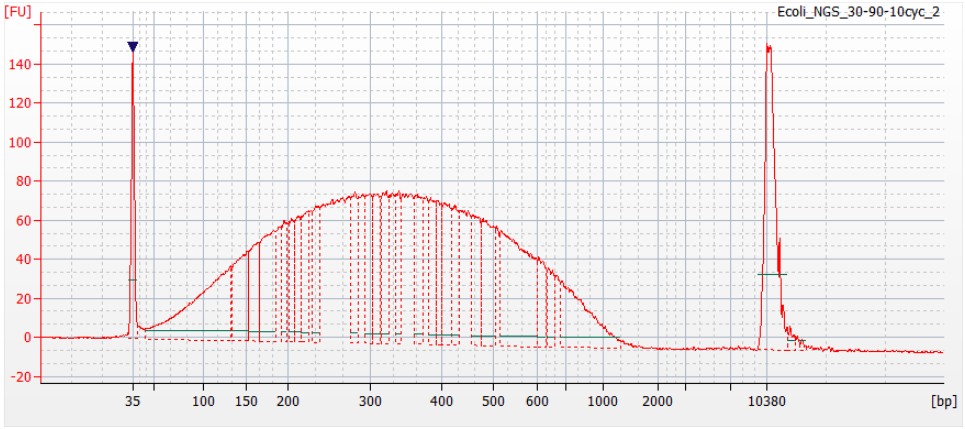
DNA fragmented with the Bioruptor™ system

Figure 4 Bioanalyzer™ instrument analysis of genomic DNA fragmented for 400-base-read libraries



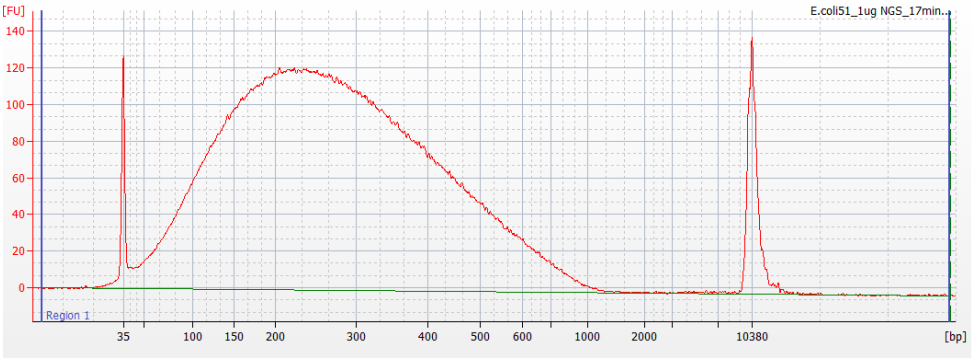
1 µg of *E. coli* DH10B DNA was fragmented with the Bioruptor™ NGS System for 20 minutes. Analysis was with the Agilent™ High Sensitivity DNA Kit. Peaks at 35 bp and 10380 bp represent low- and high-molecular weight markers.

Figure 5 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 bp and 10380 bp represent low- and high-molecular weight markers.

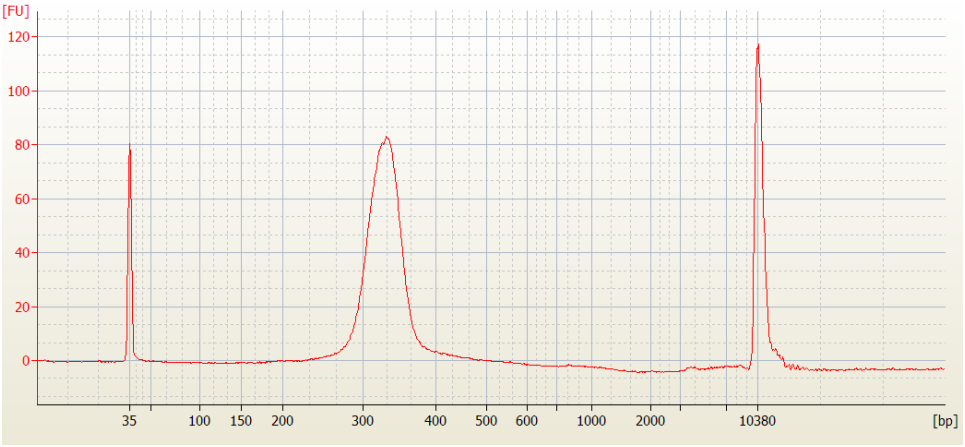
Figure 6 Bioanalyzer™ instrument analysis of genomic DNA fragmented for 100- and 200-base-read libraries



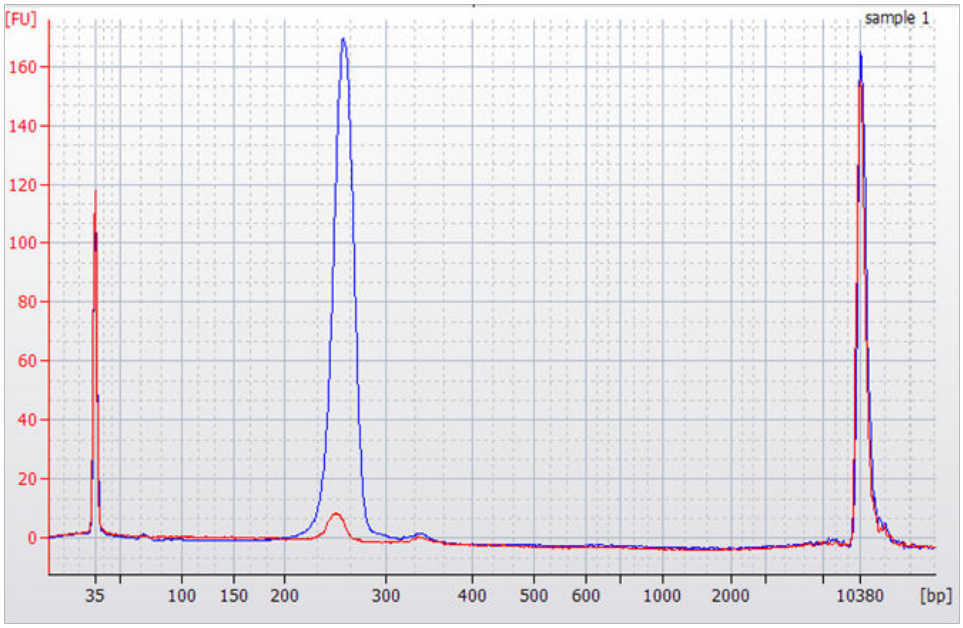
1 µg of *E. coli* DH10B DNA was fragmented with the Bioruptor™ NGS System for 17 minutes. Analysis was with the Agilent™ High Sensitivity DNA Kit. Peaks at 35 bp and 10380 bp represent low- and high-molecular weight markers.

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

Figure 7 Bioanalyzer™ instrument analysis of an unamplified 200-base-read library

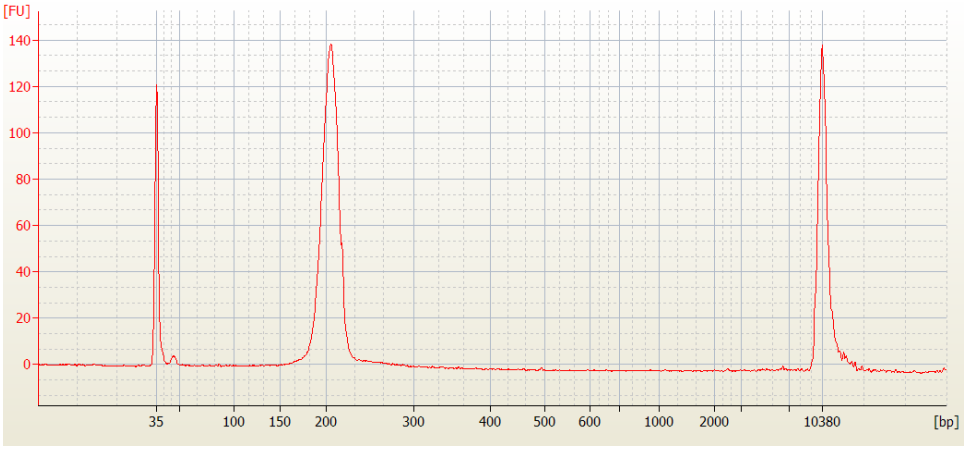


Panel A, 1 µg of *E. coli* DH10B DNA was fragmented with the Ion Shear™ Plus Reagents with a reaction time adjusted for 200-base-read libraries. An unamplified library was prepared from the fragmented DNA and size-selected with the 2% E-Gel™ SizeSelect™ II Agarose Gel for 200-base-read libraries (330-bp 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 2% E-Gel™ SizeSelect™ II 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 bp and 10380 bp represent low- and high-molecular weight markers.

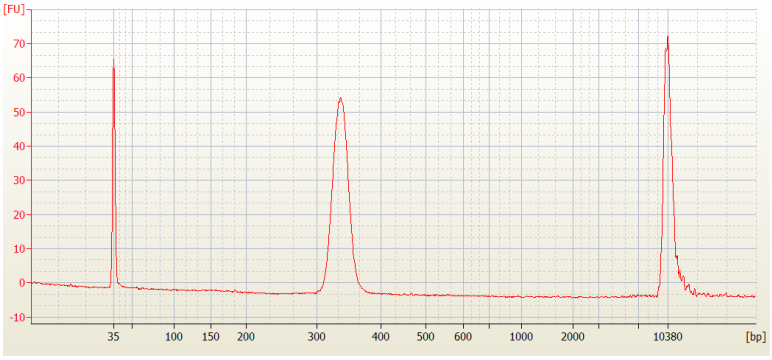
Figure 8 Bioanalyzer™ instrument analysis of an unamplified 100-base-read library



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 2% E-Gel™ SizeSelect™ II Agarose Gel 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 bp and 10380 bp represent low- and high-molecular weight markers.

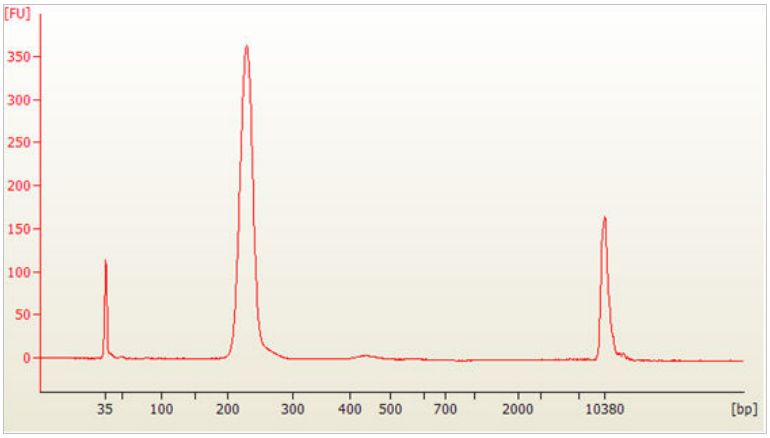
DNA fragmented with Ion Xpress™ Plus Fragment Library Kit and size-selected with the Pippin Prep™ instrument

Figure 9 Bioanalyzer™ instrument analysis of an unamplified 200-base-read library



1 µg of *E. coli* DH10B DNA was fragmented with the Ion Xpress™ Plus Fragment Library Kit 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 bp and 10380 bp represent low- and high-molecular weight markers.

Figure 10 Bioanalyzer™ instrument analysis of a 150-base-read library for the Ion Proton™ System

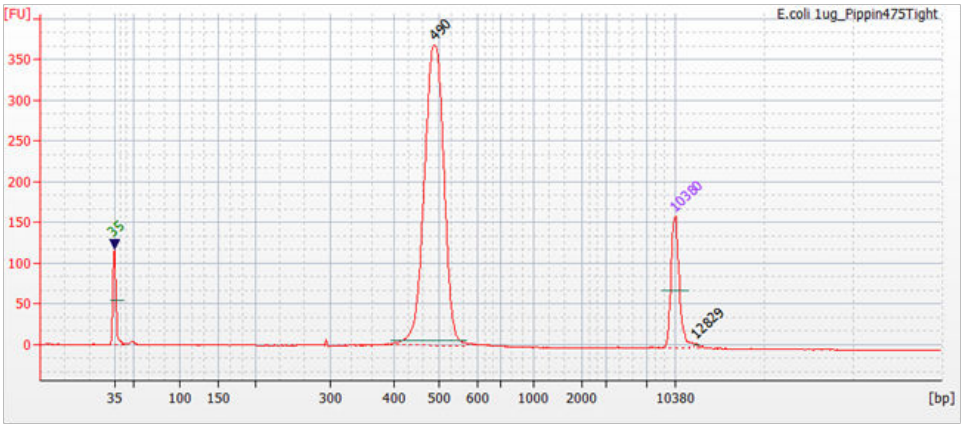


1 µg of human gDNA was fragmented with the Ion Xpress™ Plus Fragment Library Kit for 40 minutes. The fragmented DNA was size-selected with the Pippin Prep™ instrument for 150-base-read libraries (220-bp length) and then amplified, as described in this user guide. Analysis of a final library aliquot diluted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

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

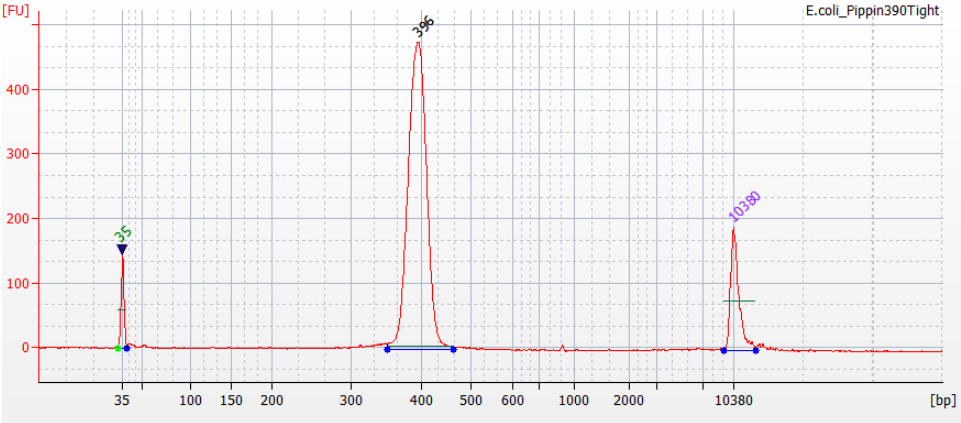
Note: For all traces, an aliquot of the final library (1:10 dilution) was analyzed with the Agilent™ High Sensitivity DNA Kit. Peaks at 35 bp and 10380 bp represent low- and high-molecular weight markers.

Figure 11 Bioanalyzer™ instrument analysis of a 400-base-read library (490-bp length)



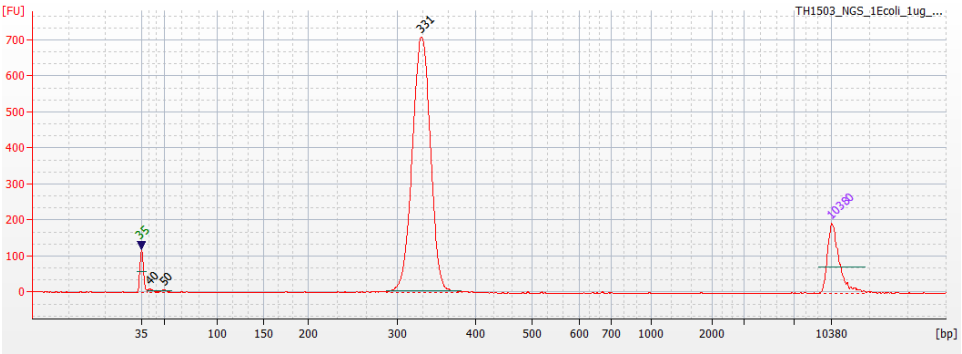
1 µg of *E. coli* DH10B DNA was fragmented with the Bioruptor™ NGS System.

Figure 12 Bioanalyzer™ instrument analysis of a 300-base-read library (390-bp length)



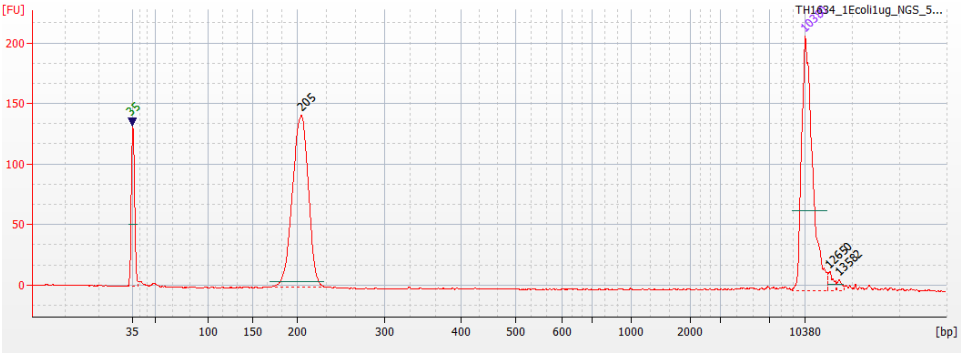
1 µg of *E. coli* DH10B DNA was fragmented with the Bioruptor™ NGS System for 20 minutes.

Figure 13 Bioanalyzer™ instrument analysis of a 200-base-read library(1 µg, 330-bp length)



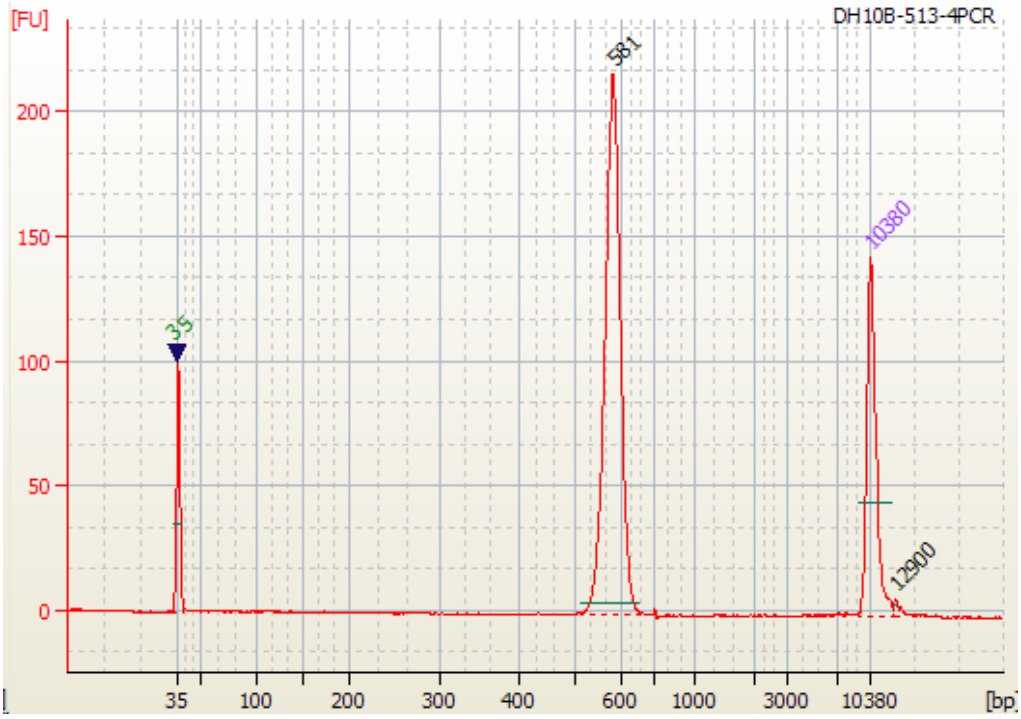
E. coli DH10B DNA was fragmented with the Bioruptor™ NGS System for 17 minutes.

Figure 14 Bioanalyzer™ instrument analysis of a 100-base-read library (1- µg, 200-bp length)



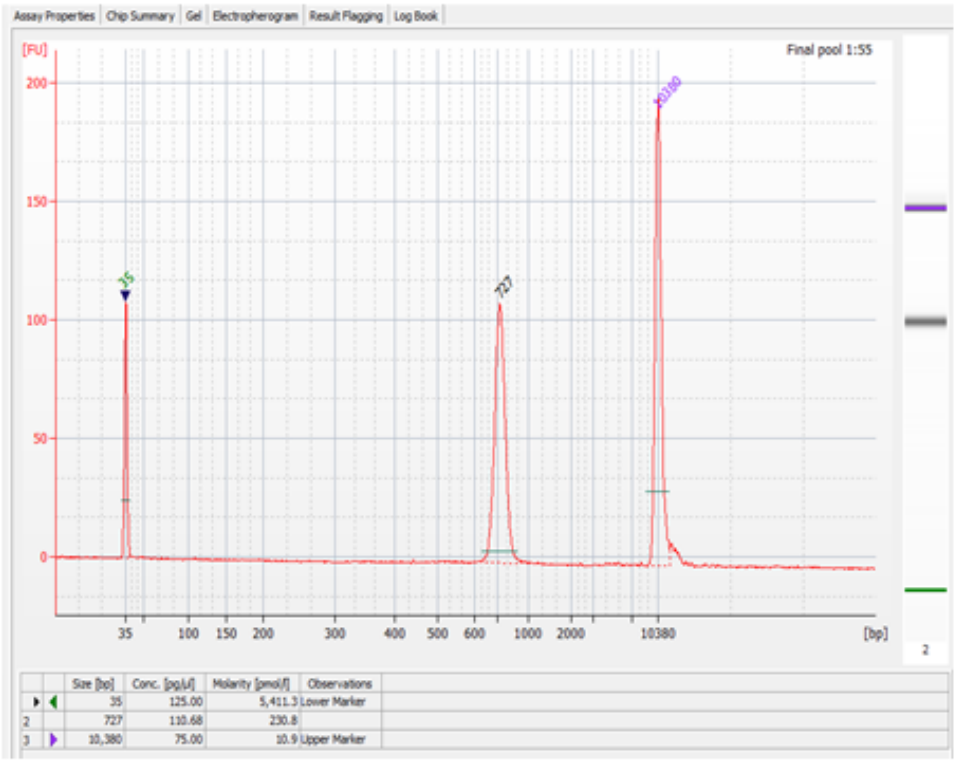
E. coli DH10B DNA was fragmented with the Bioruptor™ NGS System for 17 minutes, then size-selected with the Pippin Prep™ instrument.

Figure 15 Bioanalyzer™ instrument analysis of a 500-base-read library (1- µg, 570-bp length)



E. coli DH10B gDNA was fragmented with the Covaris™ S2 sonicator with timing adjusted for 500-base-read libraries.

Figure 16 Bioanalyzer™ instrument analysis of a 600–base–read library (1 µg)



E. coli DH10B Control 600 Library was fragmented with the Covaris™ S2 sonicator with timing adjusted for 600-base-read libraries.

Barcode discrimination

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

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

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

F

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

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2009-P.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

Related documentation

Document	Publication number
E-Gel™ SizeSelect™ Agarose Gels Quick Reference	MAN0017341
E-Gel™ Power Snap Electrophoresis System User Guide	MAN0017050
Ion 520™ & Ion 530™ ExT Kit – Chef User Guide	MAN0015805
Ion Library TaqMan™ Quantitation Kit User Guide	MAN0015802
Ion PGM™ Template IA 500 Kit User Guide	MAN0009347
Qubit™ dsDNA HS Assay Kits User Guide	MAN0002326

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

Customer and technical support

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  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

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

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.