USER GUIDE

Ion Xpress™ Plus gDNA and Amplicon Library Preparation

for use with:

Ion Xpress™ Plus Fragment Library Kit
Ion Plus Fragment Library Kit
Ion Xpress™ Barcode Adapters 1–16 Kit

Catalog Numbers 4471269, 4471252, 4471250

Publication Part Number 4471989 Rev. B
Revision Date 07 October 2011

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Publication Part Number 4471989 Rev. B
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Product information

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

Product description

To prepare high-quality libraries from genomic DNA (gDNA) or amplicons for sequencing on the Ion Personal Genome Machine™ (PGM™) System, use one or more of the following kits.

- **Ion Plus Fragment Library Kit** (Part no. 4471252). This kit includes reagents for end-repair of physically fragmented gDNA or unfragmented short (<250 bp) amplicons, and for library preparation from fragmented DNA or unfragmented short amplicons.
- **Ion Xpress™ Plus Fragment Library Kit** (Part no. 4471269): This kit includes Ion Shear™ Plus Reagents, for enzymatic fragmentation of genomic DNA or long amplicons. The kit also includes the Ion Plus Fragment Library Kit (Part no. 4471252), for subsequent library preparation from the enzymatically fragmented DNA.
- **Ion Xpress™ Barcode Adapters 1–16** (Part no. 4471250). This kit includes 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 nonbarcoded library preparation.

Use the kits to prepare nonbarcoded or barcoded libraries, as required by your application, starting material, and desired fragmentation method, described in the following table.

Application	Input DNA	Fragmentation method	Kit(s)
Whole genome resequencing Targeted resequencing*	Genomic DNA (50–100 ng or 1 µg)	Ion Shear™ Plus Reagents	Ion Xpress™ Plus Fragment Library Kit (Part no. 4471269)
	Genomic DNA (100 ng or 1 µg)	Bioruptor® System or other physical method	Ion Plus Fragment Library Kit (Part no. 4471252)
Amplicon resequencing (targeted resequencing)	Long (>400 bp) amplicon pool (100 ng)	Ion Shear™ Plus Reagents	Ion Xpress™ Plus Fragment Library Kit (Part no. 4471269)
	Short (<250 bp) amplicon pool (100 ng)	N/A	Ion Plus Fragment Library Kit (Part no. 4471252)
For sequencing libraries from multiple biological specimens in a single sequencing run	Prepare barcoded libraries using Ion Xpress™ Barcode Adapters 1–16 (Part no. 4471250) in addition to the Ion Xpress™ Plus Fragment Library Kit or Ion Plus Fragment Library Kit, as described above.		

* For targeted resequencing, unamplified whole-genome libraries are amplified and enriched for target regions of interest using a TargetSeq™ Custom Enrichment Kit.

Overview

The library preparation procedure is illustrated in **Figure 1** on page 11. **Table 1** on page 12 lists workflow options.

First, genomic DNA (gDNA) or pooled long (>400 bp) amplicons are fragmented to appropriately sized, blunt-ended DNA fragments that are ready for ligation to Ion adapters. Amplicons <250 bp in length (short amplicons) do not require fragmentation but do require end-repair for ligation.

Next, the adapter-ready DNA is ligated to Ion-compatible adapters, followed by nick repair to complete the linkage between adapters and DNA inserts. If barcoded libraries are required, substitute adapters from the Ion Xpress™ Barcode Adapters 1–16 kit at this step.

The adapter-ligated library is then size-selected for optimum length according to target read length. Short amplicon libraries do not require size selection, but the amplicons should be designed to be shorter than the median insert size for the target read length of the library.

Target read length	Median insert size	Median library size
200 bases (200 base-read library)	~260 bp	~330 bp
100 bases (100 base-read library)	~130 bp	~200 bp

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

IMPORTANT! The library preparation procedures described in this guide are compatible **only** with the Ion Xpress™ Plus Fragment Library Kit (Part no. 4471269), the Ion Plus Fragment Library Kit (Part no. 4471252), and the Ion Xpress™ Barcode Adapters 1–16 (Part no. 4471250). **Do not use** the Ion Xpress™ Fragment Library Kit (Part no. 4468987), the Ion Fragment Library Kit (Part no. 4466464), or the Ion DNA Barcoding 1–16 Kit (Part no. 4468654) with the procedures described in this guide.

Next steps in the PGM™ sequencing workflow

Unamplified or amplified libraries are ready for the Template Preparation procedure for clonal amplification on Ion Sphere™ Particles using one of the following methods:

- **For 200 base-read libraries**: The Ion Xpress™ Template 200 Kit (Part no. 4471253).
- **For 100 base-read libraries:**
- The Ion Xpress™ Template Kit (Part no. 4469001) for non-automated template preparation.
- The automated Ion OneTouch™ System (Part no. 4470001).

Alternatively, use unamplified library prepared from 1 µg of genomic DNA and the Ion TargetSeq™ Custom Enrichment kit workflow for amplification and enrichment for libraries with genomic regions of interest. Refer to the *Ion TargetSeq™ Custom Enrichment Kit User Guide* (MAN0005033).

Other library preparation options

Visit the Ion Community at **http://ioncommunity.iontorrent.com/** and **www.appliedbiosystems.com/iontorrent** for information about other methods and kits for preparation of Ion libraries, including the following options:

- Libraries from total or small RNA
- Amplicon libraries by fusion PCR
- Automated library preparation
- Mate-paired library preparation

Kit components and storage conditions

The Ion Xpress™ Plus Fragment Library Kit includes the Ion Shear™ Plus Reagents Kit and the Ion Plus Fragment Library Kit. The kit contains two boxes. Supplied reagents are sufficient for preparation of up to 20 libraries at 100 ng input, and up to 10 libraries at 1 µg input.

The Ion Plus Fragment Library Kit can be ordered alone. It contains one box. Supplied reagents are sufficient for preparation of up to 20 libraries at 100 ng input, and up to 10 libraries at 1 µg input.

Ion Xpress™ Plus Fragment Library Kit (Part no. 4471269)				
Ion Shear™ Plus Reagents Kit (Part no. 4471248)				
Components	Cap color	Quantity	Volume	Storage
Ion Shear™ Plus 10X Reaction Buffer	Clear	2 tubes	2 X 50 µL	–30°C to –10°C
Ion Shear™ Plus Enzyme Mix	Clear	2 tubes	2 X 100 µL	
Ion Shear™ Plus Stop Buffer	Clear	2 tubes	2 X 50 µL	
Ion Plus Fragment Library Kit (Part no. 4471252)				
Component	Cap color	Quantity	Volume	Storage
5X End Repair Buffer*	Red	1 tube	400 µL	–30°C to –10°C
End Repair Enzyme†	Orange	1 tube	20 µL	
10X Ligase Buffer	Yellow	1 tube	200 µL	
DNA Ligase	Blue	1 tube	40 µL	
Nick Repair Polymerase	Clear	1 tube	160 µL	
dNTP Mix	Violet	1 tube	40 µL	
Adapters	Green	1 tube	100 µL	
Platinum® PCR SuperMix High Fidelity	Black	2 tubes	1 mL	
Library Amplification Primer Mix	White	1 tube	100 µL	
Low TE	Clear	2 tubes	1.25 mL	Room temperature (15°C to 25°C) or –30°C to –10°C

* 5X End Repair Buffer and End Repair Enzyme are required only for physically fragmented gDNA and short amplicons.

The Ion Xpress™ Barcode Adapters 1–16 kit contains one box. Supplied reagents are sufficient for preparation of up to 10 libraries per barcode (10 × 16 libraries) for 100 ng input, or 2 libraries per barcode for 1 µg input. Barcoded library preparation also requires the Ion Xpress™ Plus Fragment Library Kit or the Ion Plus Fragment Library Kit.

Ion Xpress™ Barcode Adapters 1–16 (Part no. 4471250)				
Component	Cap color/label	Quantity	Volume	Storage
Ion Xpress™ P1 Adapter	Violet/—	1 tube	320 µL	–30°C to –10°C
Ion Xpress™ Barcode X	White/X	16 tubes one per barcode	20 µL each	

* X = 1 through 16

Required materials and equipment

Required for preparation of libraries from genomic DNA or amplicon DNA

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

✓	Description	Supplier	Part number	Quantity
	Agencourt® AMPure® XP Kit	Beckman Coulter	A63880 or A63881	1
	DynaMag™-2 magnet (magnetic rack)	Life Technologies (Invitrogen)	123-21D	1
	Agilent 2100 Bioanalyzer™ instrument	Agilent	G2939AA	1
	Agilent High Sensitivity DNA Kit	Agilent	5067-4626	1 kit
	1.5-mL LoBind Tubes	Eppendorf	022431021	1 box
	0.2-mL PCR tubes	MLS*	N/A	—
	Microcentrifuge	MLS	N/A	1
	Thermal cycler	MLS	N/A	1
	Vortex mixer	MLS	N/A	1
	Pipettors 1–1000 µL	MLS	N/A	1 each
	Nuclease-Free Water	Life Technologies (Applied Biosystems)	AM9932	1000 mL
	Optional: Ion Library Quantitation Kit (required for quantitation of unamplified libraries)	Life Technologies (Applied Biosystems)	4468802	1 kit
	Optional: Ion Control Materials 200 Kit	Life Technologies (Applied Biosystems)	4471249	1 kit
	Optional: 10 mM Tris, pH 7.5–8.5	MLS*	N/A	
	Optional: RNase I (100 units/µL)	Life Technologies (Invitrogen)	AM2294 AM2295	10,000 units 25,000 units
	Optional: PureLink® Genomic DNA Kit (for cleanup after optional RNase treatment)	Life Technologies (Invitrogen)	K1820-01 K1820-02 K1820-03	50 preps 250 preps 4 × 96-well plates

* Major laboratory supplier

Required for physical fragmentation of genomic DNA

Use the BioRuptor® Standard or BioRuptor® NGS Sonication System, or another system such as the Covaris® S220 System for physical fragmentation of gDNA.

✓	Description	Supplier	Part number	Quantity
	BioRuptor® Standard Sonication System with accessories for 12 x 0.5 mL tubes (Microtube Attachment & Gearplate)	Ion Torrent *or* Diagenode	4465622* UCD-200 TS*	1 1
	BioRuptor® NGS Sonication System with accessories for 12 x 0.5 mL tubes (Microtube Attachment & Gearplate)	Diagenode	UCD-600 TS	1
	Bioruptor® NGS 0.65-mL MicroTubes for DNA Shearing	Diagenode	WA-005-0500	500 tubes

* These part numbers include a soundproof box.

Required for size-selection of genomic DNA or long amplicon libraries

Use the E-Gel® SizeSelect™ 2% Agarose Gel or the Pippin Prep™ instrument for library size-selection.

✓	Description	Supplier	Part number	Quantity
	E-Gel® iBase™ unit and E_Gel® Safe Imager™ transilluminator combo kit, for library size selection	Life Technologies (Invitrogen)	G6465	1
	E-Gel® SizeSelect™ 2% Agarose, required for E-Gel® size selection method	Life Technologies (Invitrogen)	G6610-02	10/pack
	50 bp DNA ladder (1 µg/µL), required for E-Gel® size selection method	Life Technologies (Invitrogen)	10416-014	1
	Pippin Prep™ instrument, for library size-selection	Life Technologies (Applied Biosystems)	4471271	1
	2% Agarose Gel Cassettes for the Pippin Prep™ instrument	Life Technologies	4472170	10 cassettes

Required for preparation of libraries from amplicon DNA

✓	Description	Supplier	Catalog number	Qty
	Forward and reverse target-specific PCR primers for each amplicon	MLS*	N/A	10 µM stock each primer
	0.2-mL PCR strip tubes *or* 96-well PCR plate	MLS	N/A	As needed
	MicroAmp® Clear Adhesive Film	Life Technologies (Invitrogen)	4306311	100 films
	Agencourt® SPRIPlate 96R Magnet Plate *or* Magna-Sep™ 96 Magnetic Particle Separator	Beckman Coulter Life Technologies (Invitrogen)	A32782 K1585-96	1 each

* Major laboratory supplier

Procedure overview and workflow options

Figure 1 Ion genomic DNA and amplicon library preparation overview

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

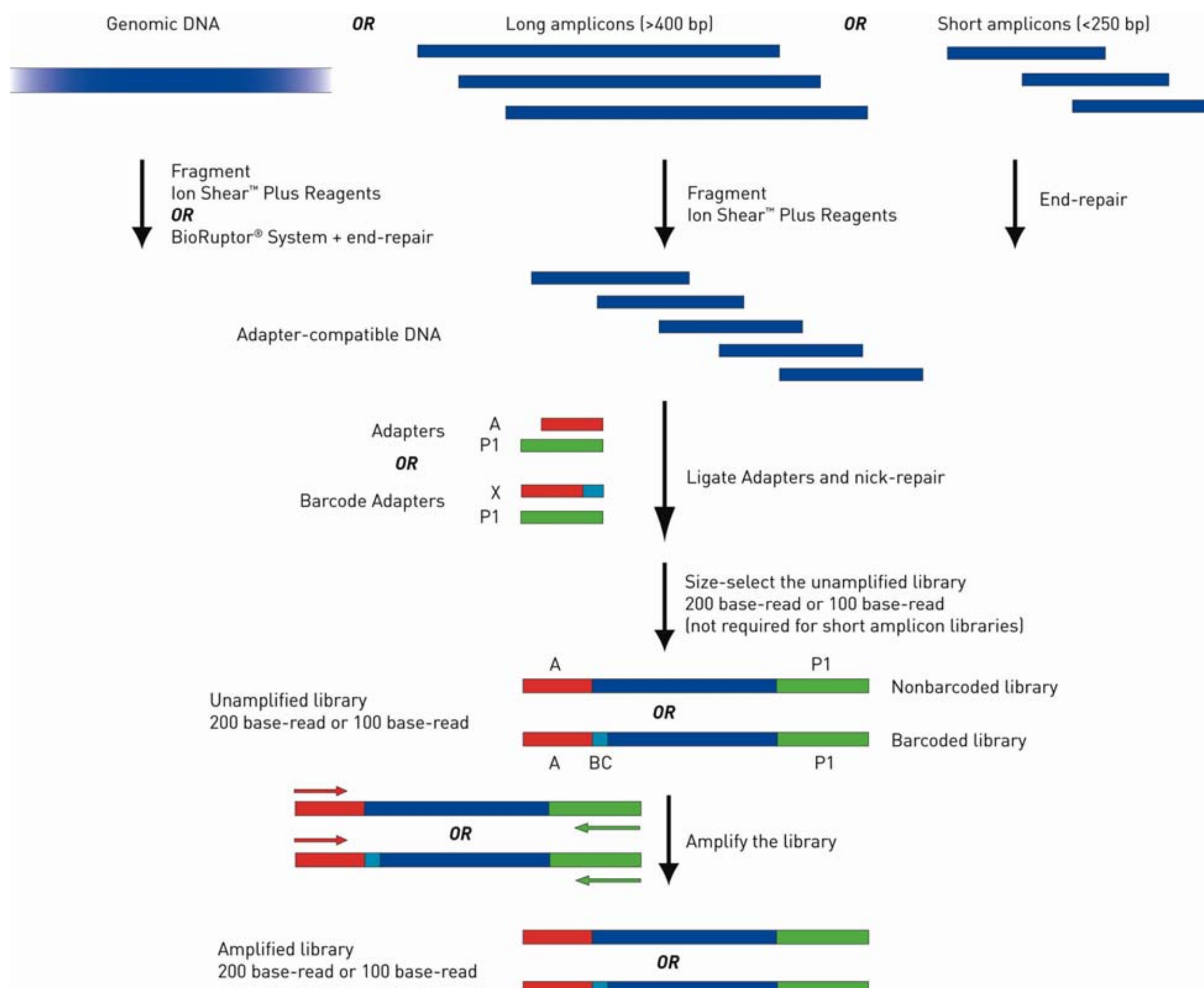


Table 1 Example workflows

By choosing the Ion Shear™ Reagents and E-Gel® SizeSelect™ Gel, an unamplified 200 base-read library can be prepared in less than 2 hours. Examples of other workflow options for fragmentation, size selection, and amplification are listed below. Refer to **Table 2** for a complete list of workflow options and features.

Input DNA	gDNA or long amplicons	gDNA	gDNA	Short amplicons (<250 bp)
Fragmentation and/or end-repair	Ion Shear™ Reagents No end-repair Pages 14–19	BioRuptor® NGS + end-repair Pages 19–22	BioRuptor® 200 + end-repair Pages 19–22	No fragmentation End-repair only Pages 24–25
Ligation, nick repair, and purification	Pages 27–31			
Size selection	E-Gel® SizeSelect™ Gel Pages 31–34	E-Gel® SizeSelect™ Gel Pages 31–34	Pippin Prep™ instrument Pages 34–38	Not required
Time to unamplified library	~100–120 min for 200 base-read library	~120 min	~260 min	~80 min
Amplification + purification	Pages 38–40			
Time to amplified library	~140 min	~160 min	~300 min	~120 min

Table 2 Workflow options

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

Fragmentation and end-repair options to prepare adapter-compatible DNA			40–130 min
Input	gDNA, 50–100 ng and 1 µg Long amplicons (>400 bp), 100 ng	gDNA, 100 ng and 1 µg	Short amplicons (<250 bp), 100 ng
Method	Enzymatic fragmentation: Ion Shear™ Plus Reagents	Bioruptor® System fragmentation + end-repair:	No fragmentation; end-repair only
Features	Fragmentation profile tuned by reaction time No end-repair	Same fragmentation conditions for 200 base-read and -100 base-read libraries	<250 bp amplicons: 200 base-read* <150 bp: 100 base-read libraries*
Time	~40 min: 200 base-read libraries* ~65 min: 100 base-read libraries*	~60 min: Bioruptor® NGS UCD-600 ~130 min: Bioruptor® UCD-200:	~40 min
Adapter ligation and nick-repair			~40 min

Size selection options			20–95 min
Input	Fragmented DNA (gDNA or long amplicons)		Short amplicons
Method	E-Gel® SizeSelect™ 2% Agarose	Pippin Prep™ instrument	None required
Features	Quicker Broader size distribution	Automated Tighter size distribution results in more consistent library size	—
Time	~20 min	85–95 min	—
Library amplification options			**0–40 min**
Input	1 µg gDNA	≥100 ng, gDNA or amplicons	<100 ng, gDNA or amplicons
Method	No amplification	Optional amplification	Required amplification
Features	Quicker qPCR quantitation required to quantify unamplified library	qPCR quantitation required to quantify unamplified library	qPCR or Bioanalyzer quantitation of amplified library
Time	—	~40 min	

* Library sizes are described in this table in terms of the target read length for the library.

Input DNA requirements and procedural guidelines

- **High-quality RNA-free DNA is required.** The quality of the input DNA has a significant impact on the quality of the resulting library. A number of commercially available kits are available for isolation of high molecular weight, RNA-free genomic DNA. See **Appendix A** for more information about assessing the integrity and size of your input DNA material and performing an optional RNase treatment procedure.
- First-time users may prepare a library to familiarize themselves with the fragmentation and library preparation procedures prior to using their own samples. Use 100 ng (1 µL) of the *E. coli* DH10B Control DNA supplied in the Ion Control Materials 200 Kit (Part no. 4471249) to prepare a genomic fragment library using the Ion Shear™ Plus Reagents. Use 100 ng or 1 µg (1 µL or 10 µL, respectively) to prepare a genomic fragment library using the BioRuptor® Sonication System.
- Use good laboratory practices to minimize cross-contamination of products. If possible, perform library construction in an area or room that is distinct from that of template preparation.
- When handling barcoded adapters, be especially careful not to cross-contaminate. Change gloves frequently and open one tube at a time.
- Perform all steps requiring 1.5-mL tubes with 1.5-mL Eppendorf LoBind Tubes (Eppendorf Part no. 022431021).
- Thaw reagents on ice before use, and keep enzymes at –20°C until ready to use.
- Mix reagents thoroughly before use, especially if frozen and thawed.

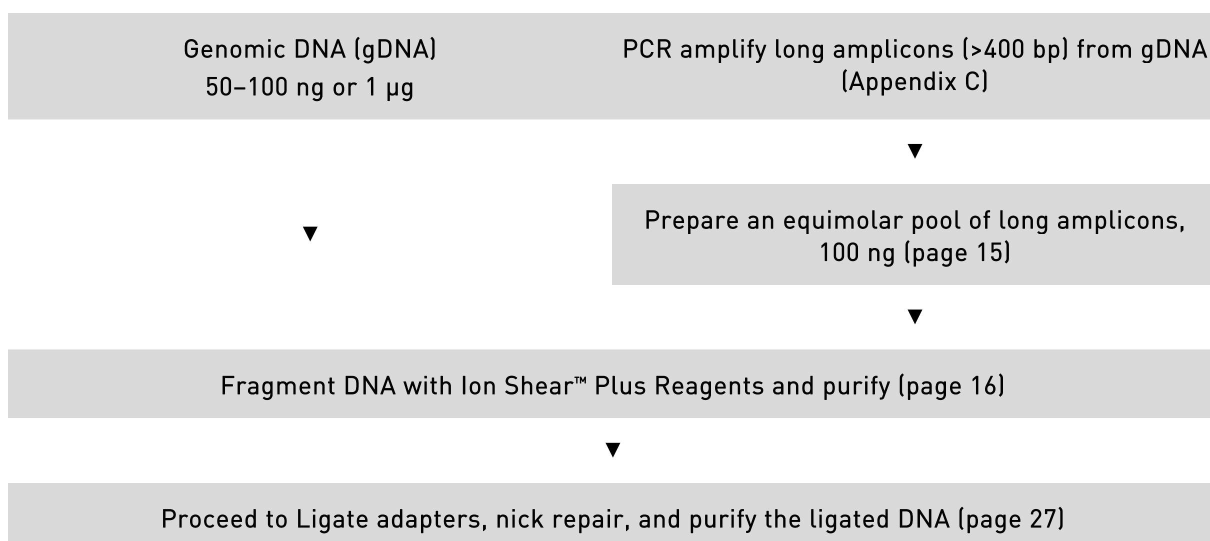
Prepare adapter-compatible DNA

This section describes three methods for preparing adapter-compatible DNA, as appropriate for your input DNA:

Input DNA	Method	Starting on...
• gDNA, 50–100 ng and 1 µg • Long amplicons (>400 bp), 100 ng	Ion Shear™ Plus Reagents	Page 14
gDNA, 100 ng and 1 µg	Bioruptor® System + end-repair	Page 19
Short amplicons (<250 bp), 100 ng	End-repair only	Page 24

Fragment gDNA or long amplicons with the Ion Shear™ Plus Reagents

This section describes conditions for enzymatic fragmentation of gDNA and long amplicons into blunt-ended fragments. No end-repair is required.



Genomic DNA

Prepare high-quality, RNA-free genomic DNA (gDNA) using any of a number of commercially available kits. Using 50–100 ng or 1 µg gDNA, proceed to **Fragment DNA with Ion Shear™ Plus Reagents** on page 16.

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

Prepare an equimolar pool of long amplicons

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

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

Required materials and equipment

- Purified amplicons >400 bp in length
- Bioanalyzer™ 2100 instrument
- Agilent High Sensitivity DNA Kit

1. Using your laboratory practices or those described in **Appendix C**, PCR amplify genomic DNA targets of interest >400 bp in length from 20–50 ng gDNA and purify the individual amplicons.
2. Prepare an equimolar pool of the amplicons at the highest possible concentration.
   a. Analyze 1 µL of each amplicon with the Agilent Bioanalyzer™ and an Agilent High Sensitivity DNA Kit. Follow the manufacturer's instructions.
   b. Use the Bioanalyzer™ software to determine the molar concentration (nmol/L) of each amplicon.
   c. Combine equimolar amounts of each amplicon. If you dilute the amplicons before pooling, use Nuclease-free Water or 10 mM Tris, pH 7.5–8.5 to prepare the diluted amplicon stocks.
3. Calculate the combined concentration of the pooled amplicons, and and convert the concentration of the pooled amplicon stock to ng/µL.

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

   STOPPING POINT (*Optional*) Store the pooled amplicon stock at –20°C. Before use, thaw the pooled amplicon stock on ice. To reduce the number of freeze-thaw cycles, store the amplicon stocks in several aliquots.

   ---

4. Proceed to **Fragment DNA with Ion Shear™ Plus Reagents and purify** in the following section.

Fragment DNA with Ion Shear™ Plus Reagents and purify

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

Library size	Median fragment size
200 base-read	200–300 bp
100 base-read	100–200 bp

Note: The Ion Shear™ Plus reaction has very good tolerance on the G+C content of a sample. However, the Ion Shear™ reaction is very sensitive to EDTA concentration, the integrity of the sample, and operator handling method. For 1 µg-input samples, it is a good practice to confirm that the reaction time is optimal for your laboratory conditions.

Materials provided in the Ion Xpress™ Plus Fragment Library Kit

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

Other materials and equipment

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

IMPORTANT! The final EDTA concentration must be ≤0.1 mM in the DNA preparation for the Ion Shear™ Plus reaction in step 3. If necessary, ethanol-precipitate the appropriate amount of the DNA preparation and resuspend in Nuclease-free Water or 10 mM Tris, pH 7.5–8 for this procedure.

Note: *(Optional)* Prepare a control sample in a separate tube, using 1 µL (100 ng) of *E. coli* DH10B Control DNA mixed with 9 µL of Nuclease-free Water or 10 mM Tris, pH 7.5–8.5.

1. Determine the volume of the input DNA, and adjust the concentration as necessary.
   - Genomic DNA (gDNA):
     - For 1 µg input: Prepare 10 µL at 100 ng/µL in Nuclease-free Water or 10 mM Tris, pH 7.5–8.5.
     - For 50–100 ng input: Determine the volume for the desired input, 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.
   - Pooled long amplicons: From the mass concentration calculated or determined during the pooling procedure, determine the volume for 100 ng.
2. Vortex the Ion Shear™ Plus 10X Reaction Buffer and the Ion Shear™ Plus Enzyme Mix each for 5 seconds, pulse-spin to bring the contents to the bottom of the tubes, and place on ice.

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

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

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

Component	Volume by input DNA		
	gDNA		Long amplicons
	50–100 ng	1 µg	100 ng
gDNA, 50–100 ng	*Y* µL	—	—
gDNA, 100 ng/µL	—	10 µL	—
Pooled long amplicons, 100 ng	—	—	*Y* µL
Ion Shear™ Plus 10X Reaction Buffer	5 µL	5 µL	5 µL
Nuclease-free Water	35 – *Y* µL	25 µL	35 – *Y* µL
Total	**40 µL**	**40 µL**	**40 µL**

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

   The total reaction volume is 50 µL.

5. Using a P100–P200 pipettor set at a 40-µL volume, mix the reaction by rapidly pipetting up and down 8–10 times. **Do not mix by vortexing and avoid creating bubbles.**

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

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

Median fragment size	Reaction time	Optimization range
200–300 bp (200 base-read library)	15 minutes	5–30 minutes
100–200 bp (100 base-read library)	40 minutes	30–60 minutes

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

8. Purify the fragmented DNA:

   IMPORTANT! Use **freshly prepared 70% ethanol** (1 mL plus overage per sample) for the next steps. A higher percentage of ethanol causes inefficient washing of smaller-sized molecules. A lower percentage of ethanol could cause sample loss.

   a. Add 99 µL of Agencourt® AMPure® XP Reagent (1.8× sample volume) to the sheared DNA sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate the mixture at room temperature for 5 minutes.

b. Pulse-spin and place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution is clear of brown tint when viewed at an angle. Carefully remove and discard the supernatant without disturbing the bead pellet.

c. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.

d. Repeat step 8.c for a second wash.

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

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

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

h. Pulse-spin and place the tube in the magnetic rack for at least 1 minute until the solution clears. **Transfer the supernatant** containing the eluted DNA to a new 0.2-mL PCR tube without disturbing the pellet.

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

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

Volume by input DNA	
50–100 ng	1 µg
1 µL	1 µL of 1:10 dilution*

* Prepare the dilution in Nuclease-free Water

Confirm the desired DNA fragment size range as follows:

Target median fragment size	Fragment size range
200–300 bp (200 base-read library)	100–700 bp
100–200 bp (100 base-read library)	50–500 bp

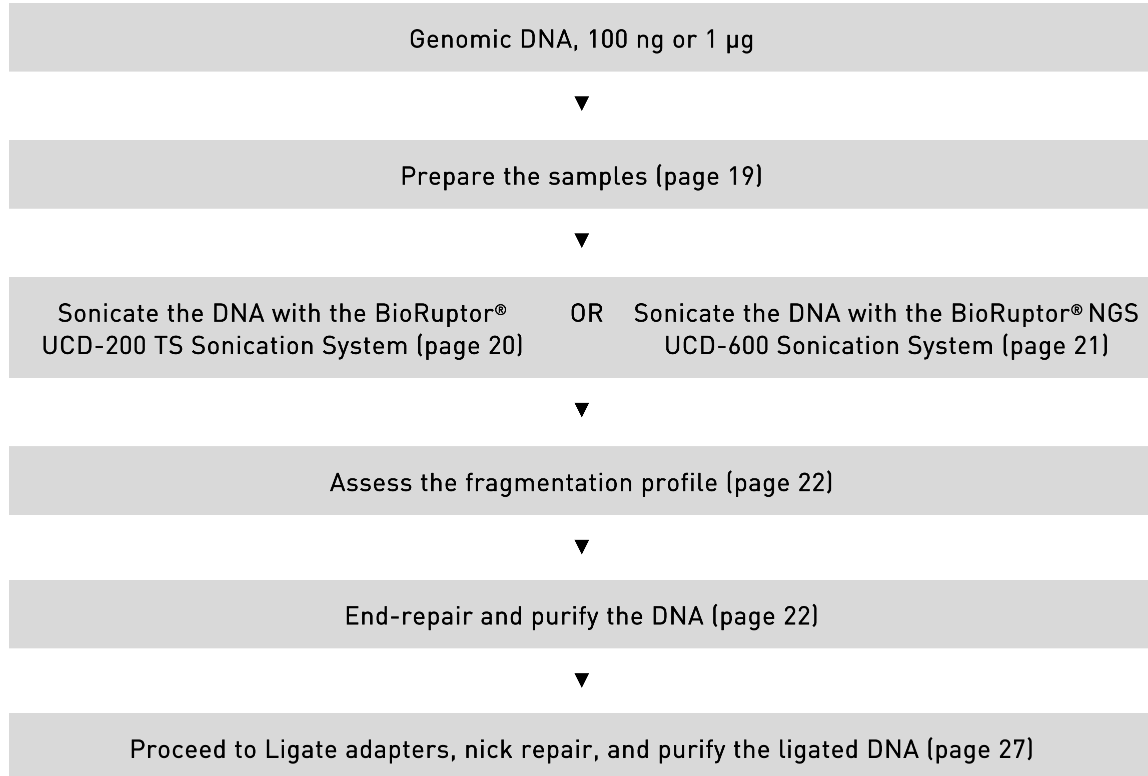
See **Figures 3 and 4** on page 47 for example traces.

STOPPING POINT (*Optional*) Store the DNA at –20°C.

10. Proceed to **Ligate Adapters, nick repair, and purify the ligated DNA** starting on page 27.

Fragment gDNA with the BioRuptor® Sonication System, end-repair, and purify

This section describes conditions for use of the BioRuptor® UCD-200 *or* the BioRuptor® NGS UCD-600 Sonication System (equipped with an adapter for 0.65-mL tubes) to shear genomic DNA by ultrasonic fragmentation. The fragmentation profile is such that 100 base-read or 200 base-read libraries can be prepared by adjusting the downstream size selection of the library molecules. The fragmented DNA is end-repaired and purified to prepare for ligation to Ion adapters.



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

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

Prepare the samples

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

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

---

2. (*Optional*) In a separate tube, prepare a control sample of 100 ng or 1 µg of E. *coli* DH10B Control DNA in 50 µL Low TE. Keep the sample on ice.
3. Process up to 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 **Sonicate the DNA with the BioRuptor® UCD-200 TS Sonication System** in the following section or to **Sonicate the DNA with the BioRuptor® UCD-600 NGS Sonication System** on page 21.

Sonicate the DNA with the BioRuptor® UCD-200 TS Sonication System

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

Interval 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 sonicate for 15 minutes:

4. Repeat Step 3 ***four*** times, for a total of 5 cycles of 15 minutes each (75 minutes total). Between each cycle, remove 1 cm (150 mL) of the water from the BioRuptor® tank and add 250 mL of crushed ice to the Fill Line.

Note: During sonication, the DNA solution might spread along the walls of the tube. This is typical and does not affect the shearing. Do not spin down the liquid between the cycles.

5. Remove the tubes from the rotor and store on ice.
6. Proceed to **Assess the fragmentation profile** on page 22.

Sonicate the DNA with the BioRuptor® UCD-600 NGS Sonication System

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

Time ON/OFF	• ON (sonication time): **30 seconds** • OFF (cool-down time): **30 seconds**
Cycle number	17
Intensity settings button	H (High)

2. Fill the BioRuptor® Sonication System to just above the Fill Line with water.
3. Switch on the Bioruptor® water cooler and set the temperature to **4°C**.
4. After the set temperature reaches 4°C, insert the rotor containing tubes into the sonicator and press **Start**. Bioruptor® Running will display on the screen. The total sonication time is 17 minutes.

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

5. Remove the tubes from the rotor and store on ice.
6. Proceed to **Assess the fragmentation profile** in the following section.

Assess the fragmentation profile

1. Analyze an aliquot of the fragmented DNA as described below to confirm a fragment size range between 50–500 bp, with a peak around 200 bp (see **Figure 5** on page 48 for an example trace).

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

2. Proceed immediately to the next step, end-repair, in the following section.

End-repair and purify the DNA

Materials provided in the Ion Plus Fragment Kit

- 5X End Repair Buffer
- End Repair Enzyme

Other materials and equipment

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

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

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

100 ng input	1 µg input
79 µL	158 µL

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

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

3. Incubate the end-repair reaction for 20 minutes at room temperature.
4. Purify the end-repaired DNA 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.

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

100 ng input	1 µg input
180 µL	360 µL

b. Pulse-spin and place the sample tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution clears. Remove and discard the supernatant without disturbing the bead pellet.

c. Without removing the tube from the magnet, dispense 500 µL of freshly prepared 70% ethanol to the sample. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.

d. Repeat step 4.c for a second wash.

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

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

g. Remove the tube from the magnet, and add 25 µL of Low TE to the sample. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.

h. Pulse-spin and place the tube in the magnetic rack for at least 1 minute. After the solution clears, **transfer the supernatant** containing the eluted DNA to a new 1.5-mL Eppendorf® LoBind Tube without disturbing the pellet.

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

STOPPING POINT (*Optional*) Store the DNA at –20°C.

5. Proceed to **Ligate Adapters and nick repair, and purify the ligated DNA** on page 27.

Pool, end-repair, and purify short amplicons

This section describes pooling and end-repair of short (<250 bp) amplicons to prepare them for ligation to Ion adapters.

PCR amplify genomic DNA (<250 bp amplicons) (Appendix C)

Prepare an equimolar pool of short amplicons, 10–100 ng total (this page)

End-repair and purify the amplicons (page 25)

Proceed to Ligate adapters, nick repair, and purify the ligated DNA (page 27)

Prepare an equimolar pool of short amplicons

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

Required materials and equipment

- Bioanalyzer™ 2100
- Agilent High Sensitivity DNA Kit

1. Using your laboratory practices or those described in **Appendix C**, PCR amplify genomic DNA targets of interest ≤250 bp in length from 20–50 ng gDNA and purify the individual amplicons. Use Nuclease-free Water for the final amplicon elution or resuspension.
2. Prepare an equimolar pool of amplicon DNA at the highest possible concentration.
   a. Analyze 1 µL of each amplicon product with the Agilent Bioanalyzer™ and an Agilent High Sensitivity DNA Kit. Follow the manufacturer's instructions.
   b. Use the Bioanalyzer™ software to determine the molar concentration (nmol/L) of each amplicon.
   c. Combine equimolar amounts of each amplicon stock. If you dilute the stocks before pooling, use Nuclease-free Water or 10 mM Tris, pH 7.5–8.5 to prepare the diluted amplicon stocks.
3. Calculate the combined concentration of the pooled amplicons, and convert the concentration of the pooled amplicon stock to ng/µL.

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

STOPPING POINT (*Optional*) Store the pooled amplicon stock at –20°C. Before use, thaw the amplicon stock on ice. To reduce the number of freeze-thaw cycles, store the amplicon stocks in several aliquots.

End-repair and purify the amplicons

Materials provided in the Ion Plus Fragment Kit

- 5X End Repair Buffer
- End Repair Enzyme

Other materials and equipment

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

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

1. Prepare 10–100 ng of the short amplicon pool in a total volume 79 µL of Nuclease-free Water.
2. Mix by pipetting in a 1.5-mL LoBind Tube:

Component	Volume
Pooled short amplicons, 10–100 ng	79 µL
5X End Repair Buffer	20 µL
End Repair Enzyme	1 µL
Total	**100 µL**

3. Incubate the end-repair reaction for 20 minutes at room temperature.
4. Purify the end-repaired DNA with the Agencourt® AMPure® XP Kit:

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

   a. Add 180 µL of Agencourt® AMPure® SP Reagent (1.8× sample volume) to the sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate at room temperature 5 minutes.

   b. Pulse-spin and place the sample tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution clears. Remove and discard the supernatant without disturbing the bead pellet.

   c. Without removing the tube from the magnet, dispense 500 µL of freshly prepared 70% ethanol to the sample. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.

   d. Repeat step 4.c for a second wash.

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

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

g. Remove the tube from the magnet, and add 25 µL of Low TE to the sample. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.

h. Pulse-spin and place the tube in the magnetic rack for at least 1 minute. After the solution clears, **transfer the supernatant** containing the eluted DNA to a new 1.5-mL Eppendorf® LoBind Tube without disturbing the pellet.

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

STOPPING POINT (*Optional*) Store the DNA at –20°C.

5. Proceed to **Ligate Adapters, nick repair, and purify the ligated DNA** in the following section.

Ligate adapters, nick repair, and purify the ligated DNA

For nonbarcoded libraries: Ligate Adapters and nick repair
For barcoded libraries: Ligate Ion Xpress™ P1 and Barcode Adapters and nick repair

Purify the ligated DNA

gDNA and long amplicons: Proceed to Size-select the unamplified library (page 31)	Short amplicons: Proceed to Determine if library amplification is required (page 37)

Materials provided in the Ion Plus Fragment Library Kit

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

Materials provided in the Ion Xpress™ Barcode Adapters 1–16 (for barcoded libraries)

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

Other materials and equipment

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

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

Reaction setup for non-barcoded libraries

Component	Volume by input DNA			
	gDNA		Long amplicons	Short amplicons
	50–100 ng	1 µg	100 ng	10–100 ng
Fragmented gDNA* or long amplicons**	~25 µL	~25 µL	25 µL	—
Short amplicons†	—	—	—	25 µL
10X Ligase Buffer	10 µL	10 µL	10 µL	10 µL
Adapters	2 µL	10 µL	2 µL	2 µL
dNTP Mix	2 µL	2 µL	2 µL	2 µL
Nuclease-free Water	51 µL	41 µL	51 µL	51 µL
DNA Ligase	2 µL	4 µL	2 µL	2 µL
Nick Repair Polymerase	8 µL	8 µL	8 µL	8 µL
Total	**100 µL**	**100 µL**	**100 µL**	**100 µL**

* Fragmented using the Ion Shear™ Reagents, or fragmented with the Bioruptor® System and end-repaired.
** Fragmented using the Ion Shear™ Reagents.
† End-repaired.

Note: Add **both** Ion P1Adapter and the desired Ion Xpress™ Barcode X adapter to the ligation reaction for barcoded libraries.

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

Reaction setup for barcoded libraries

Component	Volume by input DNA			
	gDNA		Long amplicons	Short amplicons
	50–100 ng	1 µg	100 ng	10–100 ng
Fragmented gDNA* or long amplicons**	~25 µL	~25 µL	25 µL	—
Short amplicons†	—	—	—	25 µL
10X Ligase Buffer	10 µL	10 µL	10 µL	10 µL
Ion P1 Adapter	2 µL	10 µL	2 µL	2 µL
Ion Xpress™ Barcode X††	2 µL	10 µL	2 µL	2 µL
dNTP Mix	2 µL	2 µL	2 µL	2 µL
Nuclease-Free Water	49 µL	31 µL	49 µL	49 µL
DNA Ligase	2 µL	4 µL	2 µL	2 µL
Nick Repair Polymerase	8 µL	8 µL	8 µL	8 µL
Total	**100 µL**	**100 µL**	**100 µL**	**100 µL**

* Fragmented using the Ion Shear™ Reagents, or fragmented with the Bioruptor® System and end-repaired.
** Fragmented using the Ion Shear™ Reagents.
† End-repaired.
†† X = Barcode chosen.

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

Stage	Temperature	Time
Hold	25°C	15 min
Hold	72°C	5 min
Hold	4°C	∞*

* The last stage is not a stopping point; continue directly to the purification step.

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

4. Purify the adapter-ligated and nick-translated DNA.

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

   a. Add the indicated volume of Agencourt® AMPure® XP Reagent to the sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, pulse-spin the tube, and incubate the mixture for 5 minutes at room temperature.
      - 200 base-read library: 140 µL (1.4× sample volume)
      - 100 base-read library: 180 µL (1.8× sample volume)

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

   c. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.

   d. Repeat step 4.c for a second wash.

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

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

   g. Remove the tube from the magnetic rack and add 20 µL of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.

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

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

STOPPING POINT (*Optional*) Store the DNA at –20°C.

5. Proceed according to the library input:

Library input	Proceed to...
Short amplicons	Determine if library amplification is required starting on page 37. The short amplicon library does not need size selection.
gDNA and long amplicons	Size-select the unamplified library in the next section.

Size-select the unamplified library

This section describes two options for size-selection:

- E-Gel® SizeSelect™ Agarose Gel, starting on page 31.
- Pippin Prep™ instrument, starting on page 34.

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

Library size	Target peak size
200 base-read	~330 bp
100 base-read	~200 bp

Visit the Ion Community at **http://ioncommunity.iontorrent.com/** for other library size-selection methods.

Size-select the library with the E-Gel® SizeSelect™ Agarose Gel (page 31)	OR	Size-select the library with the Pippin Prep™ instrument (page 34)

Proceed to: Determine if library amplification is required (page 37)

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

Start with unamplified library, nonbarcoded or barcoded, prepared and purified as described in **Ligate Adapters, nick repair, and purify the ligated DNA** starting on page 27.

Materials provided in the Ion Plus Fragment Kit

- Low TE

Other materials and equipment

- E-Gel® iBase™ unit and E-Gel® Safe Imager™ transilluminator combo kit
- E-Gel® SizeSelect™ 2% Agarose Gel
- 50 bp DNA Ladder (Part no. 10416-014; do not substitute other 50 bp ladders such as TrackIt™ 50 bp Ladder)
- Nuclease-free Water

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

1. Prepare the E-Gel® iBase™ unit and E-Gel® Safe Imager™ transilluminator combo unit:
    a. Place the iBase™ unit on top of the Safe Imager™ transilluminator, and plug the short cord from the Safe Imager™ into the power inlet of the iBase™ unit.
    b. Plug the connector of the power cord with the transformer into the Safe Imager™ transilluminator and connect the other end of the power cord to an electrical outlet.
    c. Verify that the iBase™ has the "SizeSelect™ 2%" program. If not, refer to "Downloading upgrade" from the *E-Gel® Technical Guide*.
2. Load an E-Gel® SizeSelect™ 2% Agarose gel in the E-Gel® iBase™ unit:
    a. Remove the gel from the package and gently remove the combs from the SizeSelect™ cassette.
    b. Insert the gel cassette into the E-Gel® iBase™ unit right edge first.
    c. Press firmly at the left edge of the cassette to seat the gel in the base. A steady light illuminates on the iBase™ unit when the cassette is properly inserted.
3. Load the gel **without** pre-running, using the following guidelines for the most accurate size cuts:
    - Load no more than 250 ng of the 50 bp DNA Ladder.
    - For size-selection of both 1 µg-input and 50–100 ng-input samples, we recommend running the 1 ug-input sample on one gel and the 50–100 ng-input on a separate gel.
    - If you must run both 1 µg- and 100 ng-input samples on the same gel, follow the guidelines for collection described in step 4.h as closely as possible. If both 1 µg- and 100 ng-input samples are collected at exactly the same time, the actual size of collected fragment from the 100 ng-input sample is always smaller than that of 1 µg-input sample.

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

IMPORTANT! Do not use wells #1 and #8 at either edge of the gel (the edge effect slows the sample migration, resulting in shorter fragments), and do not use the wells right next to the ladder well in the center (to avoid potential cross contamination with the ladders).

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

---

    a. **For 1 µg-input samples:** Before loading, add 20 µL of Low TE to the purified ligated DNA to bring the total volume to 40 µL.
    b. Add 20 µL of ligated DNA to the loading well (top row). Use one well for 50–100 ng-input samples.

       Note: Use two adjacent wells for 1 µg-input samples, on one side of the gel. For example, use well positions 2 and 3, or well positions 6 and 7.
    c. Dilute the 1 µg/µL 50 bp DNA Ladder in Low TE buffer to 25 ng/µL (1:40 dilution). Add 10 µL of diluted DNA ladder into the middle well, lane M. Load no more than 250 ng (10 µL of 1:40 dilution) of the 50 bp DNA Ladder.
    d. Add 25 µL of Nuclease-free Water to all empty wells in the top row.
    e. Add 25 µL of Nuclease-free Water to all the large wells in bottom row (collection wells), and add 10 µL to the center well (lane M) of the bottom row.

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

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

   b. Select **Run SizeSelect 2%** program, and set the time to the value under **Run Time to Reference Line** in the Run Time Estimation Table in the *E-Gel® SizeSelect™ Agarose Gels Quick Reference* for the appropriate band size, as described below.

      It is better to set the shorter run time if you are a novice user.

Library size	Target library length	Run Time to Reference Line
200 base-read	330 bp	12–14 minutes
100 base-read	200 bp	11–12.5 minutes

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

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

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

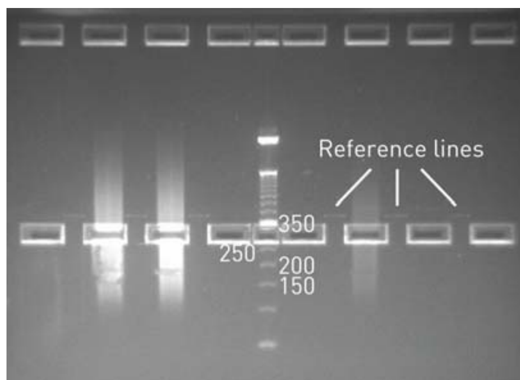
   f. Refill the collection wells to 25 µL with ~10 µL of Nuclease-free Water. The water in the wells should form a concave surface. **Do not overfill.**

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

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

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

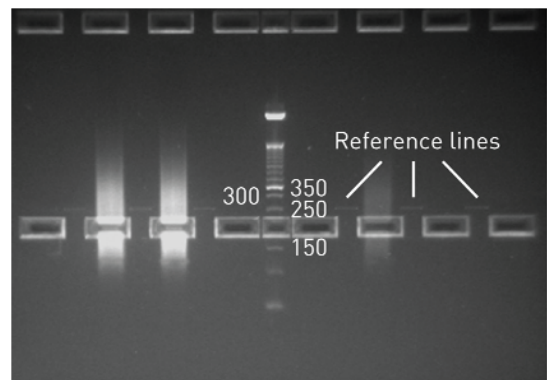
- **For 1 µg-input samples**, stop the run right before the 350 bp band is about to touch the top edge of the collection well.
- **For 50–100 ng-input samples**, stop the run when the 350 bp ladder band has just completely entered the top edge of the collection well.



200 base-read library gel

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

The 200 bp ladder band is in the middle of the collection well.



100 base-read library gel

i. Collect the solution from the collection wells using a pipette, without piercing the bottom of the well.

j. Refill the well with 10 µL Nuclease-free Water to wash the collection well, collect the solution, and pool the solutions. The total recovered volume is ~30 µL from each well.

k. **For 1 µg-input samples,** combine the recovered DNA from the two appropriate wells. The total volume is ~60 µL.

l. Dispose of the used gels as hazardous waste.

5. Proceed to **Determine if library amplification is required** on page 37.

Note: **Unamplified size-selected libraries prepared from 1 µg gDNA input** are ready to enter the Ion TargetSeq™ Custom Enrichment Kit workflow for amplification and enrichment for libraries of genomic regions of interest. Refer to the *Ion TargetSeq™ Custom Enrichment Kit User Guide* (MAN0005033).

Size-select the library with the Pippin Prep™ instrument

Start with unamplified library, nonbarcoded or barcoded, prepared and purified as described in **Ligate Adapters, nick repair, and purify the ligated DNA** starting on page 27.

Materials provided in the kit

- Low TE

Other materials and equipment

- Pippin Prep™ instrument (Part no. 4471271)
- 2% Agarose Gel Cassettes for the Pippin Prep™ instrument (Part no. 4472170)
- Nuclease-free Water
- Agencourt® AMPure® XP Kit
- Freshly prepared 70% ethanol
- Magnetic rack

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

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

   a. Unwrap the cassette, and then tip it toward the loading wells end to dislodge any air bubbles present around the elution wells and then insert the cassette into the instrument.

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

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

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

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

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

a. From the cassette type drop-down menu, choose **2% Marker B**.

b. Press **Ref Lane** (reference lane) to define lane 5 as the ladder lane.

c. Define lanes 1–4 as sample lanes, then define BP Target size for each lane used.

Library size	BP Target setting
200 base-read	315 bp
100 base-read	180 bp

d. For every sample lane, press the **Sig Mon** (signal monitoring) button to inactivate lane monitoring. Each button for an inactivated lane turns dark.

e. Set the run time for 1.5 hours.

3. Load the sample:

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

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

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

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

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

e. Remove 40 µL of Electrophoresis Buffer from one sample loading well at a time, then immediately load the entire 40-µL sample into the well.

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

4. Run the instrument:

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

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

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

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

5. Purify the size-selected DNA:

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

a. Add 108 µL of Agencourt® AMPure® beads (1.8× sample volume) to the sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, pulse-spin the tube, and incubate the mixture for 5 minutes at room temperature.

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

c. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol to the sample. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.

d. Repeat step 5.c for a second wash.

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

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

g. Remove the tube from the magnetic rack, and add the indicated volume 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.

- 1 µg input: 40 µL
- 50–100 ng input: 20 µL

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

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

6. Proceed to **Determine if library amplification is required** in the next section.

Note: **Unamplified size-selected libraries prepared from 1 µg gDNA input** are ready to enter the Ion TargetSeq™ Custom Enrichment Kit workflow for amplification and enrichment for libraries of genomic regions of interest. Refer to the *Ion TargetSeq™ Custom Enrichment Kit User Guide* (MAN0005033).

Determine if library amplification is required

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

Note: In general, 1 μg-input libraries do not require amplification, and libraries from <100 ng inputs do require amplification. However, it can be useful to follow the procedure described in this section for libraries prepared from all input amounts, especially when preparing libraries from a new sample type for the first few times.

Quantify the unamplified library by qPCR with the Ion Library Quantitation Kit (Part no. 4468802). This kit directly determines the Template Dilution Factor that gives a suitable concentration for template preparation (~26 pM, or ~15.5×10^6 molecules per μL).

1. Determine the Template Dilution Factor (TDF) for the unamplified library with the Ion Library Quantitation Kit. Follow the instructions in the *Ion Library Quantitation Kit User Guide* (Part no. 4468986), and dilute the **unamplified** library for the qPCR as follows:
   - 100 ng-input: 1:1000 dilution
   - 1 μg-input: 1:2000 dilution
2. Calculate the number of Template Preparation reactions that can be performed with the unamplified library as follows:

   No. of reactions =
   [(library volume in μL) × TDF] ÷ [volume per Template Preparation reaction in μL]

   Where the volume per Template Preparation reaction is:
   - Ion Xpress™ Template Kits (Part nos. 4471253 and 4469001): 18 μL
   - Ion OneTouch™ System (Part no. 4470001; 100 base-read libraries only): 5 μL

   If the estimated number of Template Preparation reactions is sufficient for your experimental requirements, no amplification is necessary.
3. Proceed to either amplify or further qualify the library, according to your experimental needs.

Library amplification	Proceed to...
Yes	Amplify the library on page 37
No	• **Nonbarcoded libraries:** Qualify the library on page 40 • **Barcoded libraries:** Qualify and pool barcoded libraries on page 43

Amplify and purify the library

Materials provided in the Ion Plus Fragment Library Kit

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

Other materials and equipment

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

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

Size-selection method	E-Gel® SizeSelect™ Agarose Gel		Pippin Prep™ instrument		N/A Short amplicons
Library input amount	50–100 ng	1 µg	50–100 ng	1 µg	10–100 ng
Volume, unamplified library	~30 µL	~60 µL	20 µL	40 µL	20 µL
Low TE	—	—	5 µL	10 µL	5 µL
Volume to amplification rxn (step 2)	25 µL*	50 µL*	25 µL	50 µL	25 µL

* Save the remainder for troubleshooting.

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

Component	Volume by input DNA			
	gDNA		Long amplicons	Short amplicons
	50–100 ng	1 µg	100 ng	10–100 ng
Platinum® PCR SuperMix High Fidelity	100 µL	200 µL	100 µL	100 µL
Library Amplification Primer Mix*	5 µL	10 µL	5 µL	5 µL
Size-selected unamplified library	25 µL	50 µL	25 µL	—
Short amplicon unamplified library	—	—	—	25 µL
Total	**130 µL**	**260µL**	**130 µL**	**130 µL**

* Barcoded and non-barcoded libraries use the same Library Amplification Primer Mix

3. Split the 260-µL reaction mix into two 0.2-mL PCR tubes, each containing about 130 µL.

   If desired, you can split the 130-µL reaction into two 0.2-mL PCR tubes, each containing about 65 µL, and split the 260-µL reaction mix into three 0.2-mL PCR tubes, each containing about 86 µL.

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

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

Stage	Step	Temperature	Time
Holding	Denature	95°C	5 min
Cycling*	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	70°C	1 min
Holding	—	4°C	∞

*Set the number of cycles according to the following table.

Number of cycles by library input			
gDNA or long amplicons		Short amplicons	
50–100 ng	1 µg	10 ng	100 ng
8	5	8	5

5. **For the 1 µg-input library**: Combine the previously split PCRs in a new 1.5-mL LoBind tube.
6. Purify the library DNA:

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

   a. Add the indicated volume of Agencourt® AMPure® XP Reagent (1.5× sample volume) to each sample, pipet up and down 5 times to thoroughly mix the bead suspension with the DNA, then pulse-spin and incubate the mixture for 5 minutes at room temperature.
      - 10–100 ng input: 195 µL
      - 1 µg input: 390 µL
   b. Pulse-spin and place the tube in a magnetic rack such as the DynaMag™-2 magnet for 3 minutes or until the solution is clear. Carefully remove and discard the supernatant without disturbing the pellet.
   c. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol. Incubate for 30 seconds, turning the tube around twice in the magnet to move the beads around. After the solution clears, remove and discard the supernatant without disturbing the pellet.
   d. Repeat step 5.c for a second wash.
   e. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, and carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
   f. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.
   g. Remove the tube from the magnetic rack, and add 20 µL of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down 5 times, then vortex the sample for 10 seconds, to mix thoroughly.

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

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

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

STOPPING POINT Store the library at –20°C. Before use, thaw on ice. To reduce the number of freeze-thaw cycles, store the library in several aliquots.

7. **For non-barcoded libraries**, proceed to **Qualify the library** in the next section.

   For barcoded libraries, proceed to **Qualify and pool barcoded libraries** starting on page 43.

Qualify the library

For barcoded libraries, follow **Qualify and pool barcoded libraries** starting on page 43.

Assess the size distribution of the library

Required materials and equipment

- Agilent Bioanalyzer™ instrument
- Agilent High Sensitivity DNA Kit
- *(Optional)* Low TE

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

See **Figure 6** on page 48 through **Figure 10** on page 50 for example traces.

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

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

Determine the library dilution required for Template Preparation

Quantitate the library to determine the library dilution (Template Dilution Factor) that results in a concentration within the optimized target range for Template Preparation (PCR-mediated addition of library molecules onto Ion Sphere™ Particles). The Template Dilution Factor can be used for template preparation using the Ion Xpress™ Template Kit (Part no. 4469001), the Ion Xpress™ Template 200 Kit (Part no. 4471253), or the automated Ion OneTouch™ System (Part no. 4470001).

- **Unamplified libraries:** Determine the Template Dilution Factor by qPCR with the Ion Library Quantitation Kit (Part no. 4468802).
- **Amplified libraries:** Determine the Template Dilution Factor by Bioanalyzer™ analysis *or* by qPCR.

Quantitation method	Features
Ion Library Quantitation Kit (qPCR)	• Quantitative real-time PCR (qPCR) methodology. • Direct determination of the Template Dilution Factor. • Higher precision for quantitation. A single dilution of the library, based on the Template Dilution Factor determined with the Ion Library Quantitation Kit, is usually sufficient for an optimized Template Preparation procedure. • Higher sensitivity for detection. The Ion Library Quantitation Kit is recommended for unamplified or low-yield libraries. Libraries with insufficient material for detection by Bioanalyzer™ 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 Quantiation Kit accurately quantifies the properly adapted libraries with minimal impact from background material.
Bioanalyzer™ analysis	• Determination of a molar concentration for the library, from which the Template Dilution Factor is calculated. • Concentration is part of the output of the Bioanalyzer™ 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™ analysis must be performed for optimized Template Preparation.

If you perform both procedures:

- Use the Ion Library Quantitation Kit to determine the Template Dilution Factor.
- Use Bioanalyzer ™ instrument analysis to assess the size distribution of the library.

Determine the Template Dilution Factor with the Ion Library Quantitation Kit (for unamplified or amplified libraries)

Use the Ion Library Quantitation Kit (Part no. 4468802) to directly determine the Template Dilution Factor by quantitative real-time PCR (qPCR). Follow the instructions in the *Ion Library Quantitation Kit User Guide* (Part no. 4468986).

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

Determine the Template Dilution Factor from Bioanalyzer™ analysis (amplified libraries only)

1. From the Bioanalyzer™ analysis used to assess the library size distribution, determine the molar library concentration in pmol/L using the Bioanalyzer™ software. If necessary, follow the manufacturer's instructions to perform a region analysis (smear analysis) to place the entire distribution of library molecules within a single peak.
2. Determine the Template Dilution Factor that gives a concentration of ~26 pM (~15.5 × 10^6 molecules per µL). This concentration is suitable for template preparation using either the Ion Xpress™ Template Kits or the Ion OneTouch™ System. Use the following formula:

   Template Dilution Factor = (Library concentration in pM)/26 pM

   Example: The library concentration is 10,000 pM.

   Template Dilution Factor = 10,000 pM/26 pM = 385

   Thus, 1 µL of library mixed with 385 µL of Low TE (1:385 dilution) yields approximately 26 pM (15.5 × 10^6 molecules per µL) for either the Ion Xpress™ Template Kits or the Ion OneTouch™ System procedure.

   Note: Because Bioanalyzer™ quantitation is not as precise as qPCR, when you perform the Template Preparation procedure, you will need to prepare 3 serial dilutions of the library at ½× Template Dilution Factor, Template Dilution Factor, and 2× Template Dilution Factor, to ensure that one or more dilutions are in the optimized concentration range.

Proceed to Template Preparation

The libraries are ready for the downstream Template Preparation procedure for clonal amplification on Ion Sphere™ using one of the following methods.

- **For 200 base-read libraries**: The Ion Xpress™ Template 200 Kit (Part no. 4471253). Refer to the *Ion Xpress™ Template 200 Kit User Guide* (Part no. 4471974).
- **For 100 base-read libraries**:

- The Ion Xpress™ Template Kit (Part no. 4469001) for non-automated template preparation. Refer to the *Ion Xpress™ Template Kit User Guide v2.0* (Part no. 4469004).
- The automated Ion OneTouch™ System (Part no. 4470001). Refer to the *Ion OneTouch™ Template Kit User Guide* (Part no. 4468007).

The user guides are available at the Ion Torrent users community **http://ioncommunity.iontorrent.com** and **www.appliedbiosystems.com/iontorrent**.

Qualify and pool barcoded libraries

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

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

For non-barcoded libraries, follow **Qualify the library** starting on page 40.

Assess the size distribution of individual barcoded libraries by Bioanalyzer™ analysis	
▼	▼
Amplified libraries only: Bioanalyzer™ analysis quantitation	Unamplified or amplified libraries: qPCR with Ion Library Quantitation Kit (Part no. 4468802)
▼	▼
Quantitate individual libraries with Bioanalyzer™ analysis	Determine Template Dilution Factor of individual libraries from qPCR
▼	▼
Pool equimolar amounts of individual libraries	Dilute each library by Template Dilution Factor
▼	▼
Determine Template Dilution Factor of library pool (library pool must be diluted by Template Dilution Factor)	Pool equal volumes of diluted libraries (library pool is at correct concentration for Template Preparation)

Assess the size distribution of individual barcoded libraries

Materials required for this procedure

- Agilent® Bioanalyzer™ 2100 instrument
- Agilent® High Sensitivity DNA Kit
- Low TE

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

See **Figure 6** on page 48 through **Figure 10** on page 50 for example traces. Individual barcoded libraries display the same size distributions as nonbarcoded libraries.

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

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

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

1. Use the Ion Library Quantitation Kit (Part no. 4468802) to directly determine the Template Dilution Factor by quantitative real-time PCR (qPCR) for each individual barcoded library. Follow the instructions in the *Ion Library Quantitation Kit User Guide* (Part no. 4468986).
2. Dilute each barcoded library according to its Template Dilution Factor.

   For example, if the Template Dilution Factor is 350, mix 1 µL of the final library with 349 µL of Low TE.
3. Prepare at least 20 µL of a barcoded library pool by mixing equal volumes of the diluted barcoded libraries.

   Note: The library pool is at the correct concentration for the Template Preparation procedure. No further dilution of the library pool is necessary.

   Note: The Template Dilution Factor determined with the Ion Library Quantitation Kit can be used for template preparation using the Ion Xpress™ Template Kit (Part no. 4469001), the Ion Xpress™ Template 200 Kit (Part no. 4471253), or the automated Ion OneTouch™ System (Part no. 4470001).

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

1. From the Bioanalyzer™ analysis used to assess the individual barcoded library size distribution, determine the molar concentration in pmol/L of each barcoded library using the Bioanalyzer™ software. If necessary, follow the manufacturer's instructions to perform a region analysis (smear analysis) to place the entire distribution of library molecules within a single peak.
2. Prepare an equimolar pool of barcoded libraries at the highest possible concentration.

STOPPING POINT *(Optional)* Store the library pool at –20°C. To reduce the number of freeze-thaw cycles, store the library pool in several aliquots. Thaw on ice.

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

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

4. Determine the Template Dilution Factor that gives a concentration of ~26 pM (~15.5×10^6 molecules per µL). This concentration is suitable for template preparation using either the Ion Xpress™ Template Kits or the Ion OneTouch™ System. Use the following formula:

   Template Dilution Factor = (Library pool concentration in pM)/26 pM

   Example: The library pool concentration is 10,000 pM.

   Template Dilution Factor = 10,000 pM/26 pM = 385

   Thus, 1 µL of library pool mixed with 385 µL of Low TE (1:385 dilution) yields approximately 26 pM (15.5×10^6 molecules per µL) for either the Ion Xpress™ Template Kits or the Ion OneTouch™ System procedure.

   Note: Because Bioanalyzer™ quantitation is not as precise as qPCR, when you perform the Template Preparation procedure, you will need to prepare 3 serial dilutions of the library pool at ½× Template Dilution Factor, Template Dilution Factor, and 2× Template Dilution Factor, to ensure that one or more dilutions are in the optimized concentration range.

Proceed to Template Preparation

The barcoded library pool is ready for the downstream Template Preparation procedure for clonal amplification on Ion Sphere™ using one of the following methods.

- **For 200 base-read libraries**: The Ion Xpress™ Template 200 Kit (Part no. 4471253). Refer to the *Ion Xpress™ Template 200 Kit User Guide* (Part no. 4471974).
- **For 100 base-read libraries**:

- The Ion Xpress™ Template Kit (Part no. 4469001) for non-automated template preparation. Refer to the *Ion Xpress™ Template Kit User Guide v2.0* (Part no. 4469004).
- The automated Ion OneTouch™ System (Part no. 4470001). Refer to the *Ion OneTouch™ Template Kit User Guide* (Part no. 4468007).

The user guides are available at the Ion Torrent users community **http://ioncommunity.iontorrent.com** and **www.appliedbiosystems.com/iontorrent**.

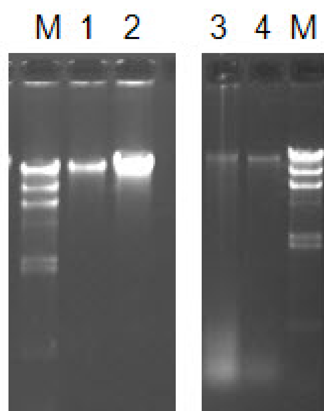
Appendix A Evaluate the quality of the genomic DNA

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 2 Examples of genomic DNA preparations

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



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

(Optional) Treat the DNA with RNase I

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

Required materials and equipment

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

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

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

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

Appendix B Bioanalyzer™ instrument analysis of library preparation

Figure 3 Bioanalyzer™ instrument analysis of genomic DNA fragmented with Ion Shear™ Plus Reagents—200 base-read

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. The fragmentation profile of pooled long amplicons is very similar. Analysis was with the Agilent High Sensitivity DNA Kit.Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

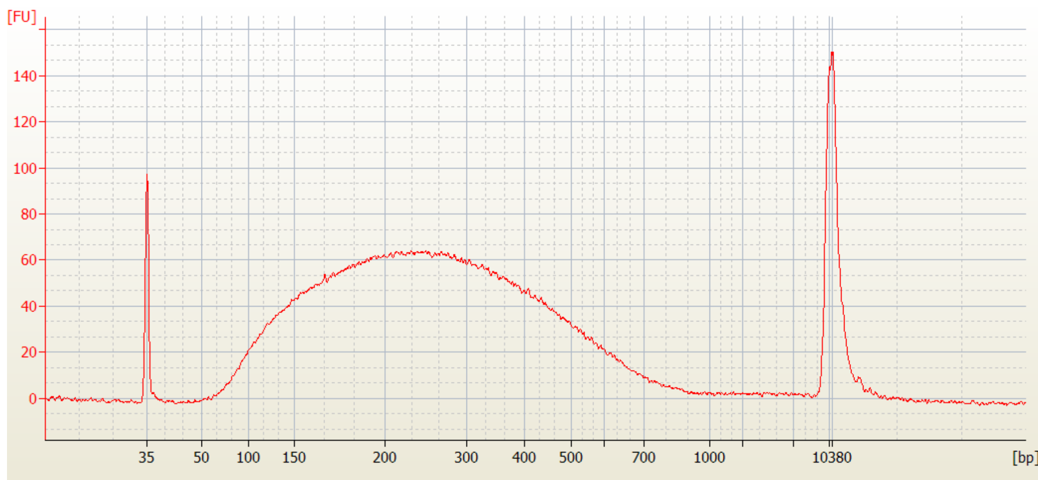


Figure 4 Bioanalyzer™ instrument analysis of genomic DNA fragmented with Ion Shear™ Plus Reagents—100 base-read

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. The fragmentation profile of pooled long amplicons is very similar. Analysis was with the Agilent High Sensitivity DNA Kit. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

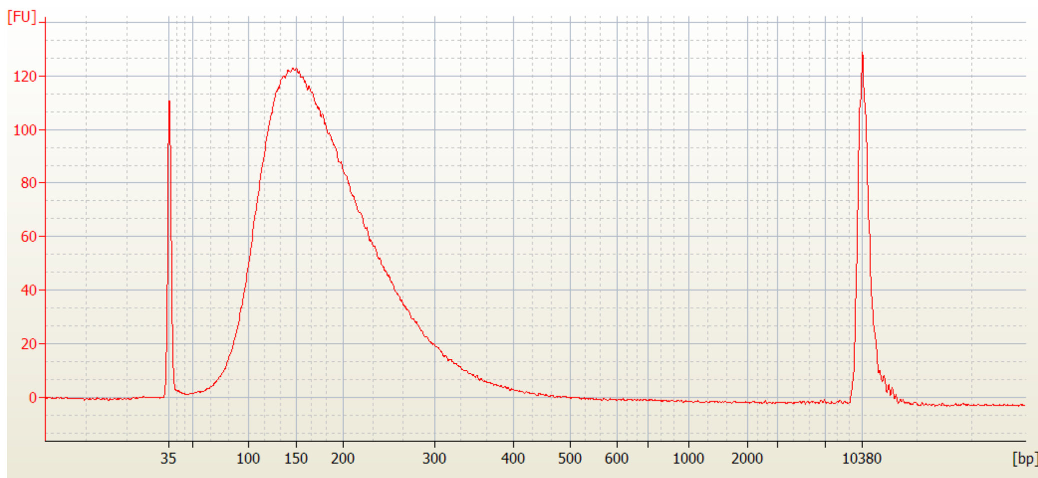


Figure 5 Bioanalyzer™ instrument analysis of genomic DNA fragmented with the Bioruptor® System

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

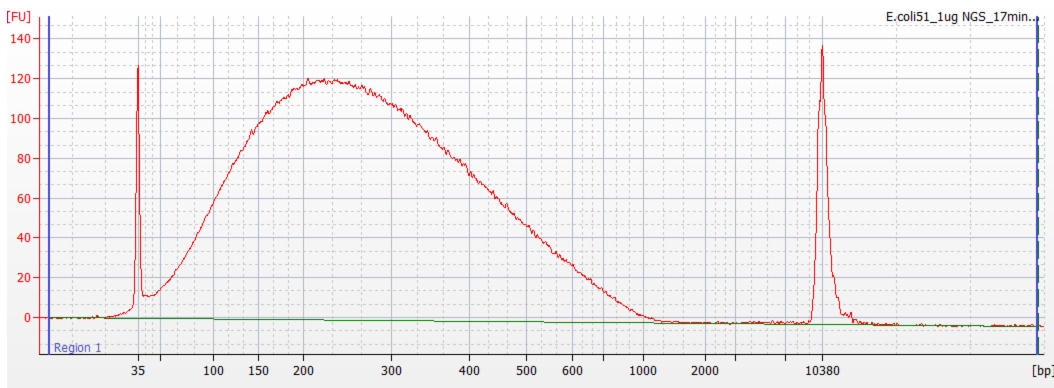


Figure 6 Bioanalyzer™ instrument analysis of an unamplified 200 base-read library—Ion Shear™ Plus Reagents and E-Gel® SizeSelect™ Agarose Gel

1 µg of *E. coli* DH10B DNA was fragmented with the Ion Shear™ Plus Reagents with a reaction time adjusted for 200 base-read libraries. An unamplified library was prepared from the fragmented DNA and size-selected with the E-Gel SizeSelect 2% Agarose for 200 base-read libraries (330 bp length), as described in this user guide. Analysis was with the Agilent High Sensitivity DNA Kit. Libraries prepared from pooled long amplicons have a very similar profile. Peaks at 35 and 10380 bp represent low- and high-molecular weight markers.

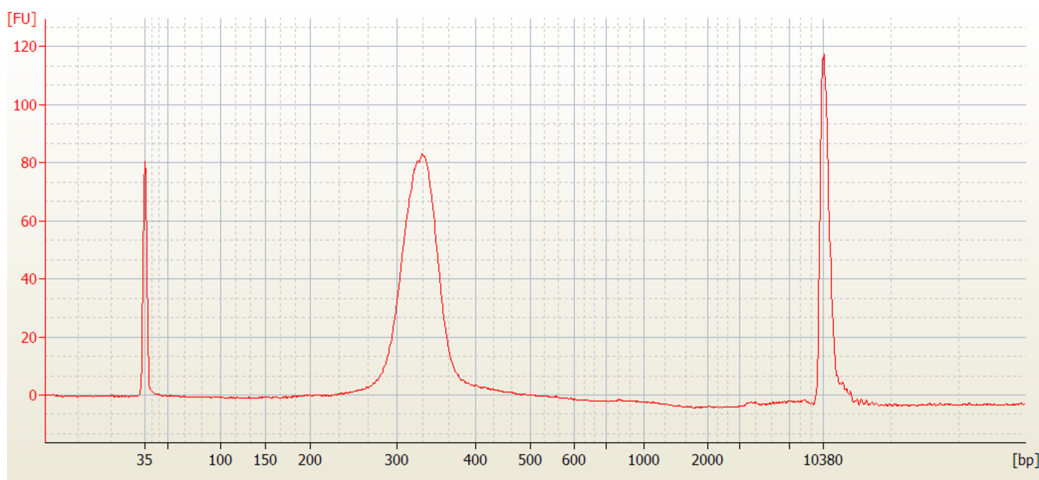


Figure 7 Bioanalyzer™ instrument analysis of an unamplified 100 base-read library— Ion Shear™ Plus Reagents and E-Gel® SizeSelect™ Agarose Gel

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

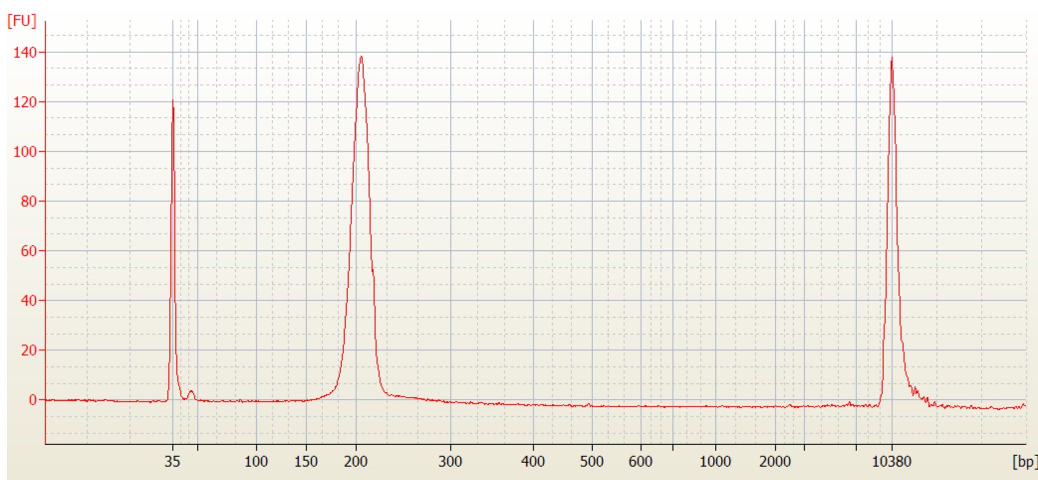


Figure 8 Bioanalyzer™ instrument analysis of an unamplified 200 base-read library— Ion Shear™ Plus Reagents and Pippin Prep™ size-selection

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

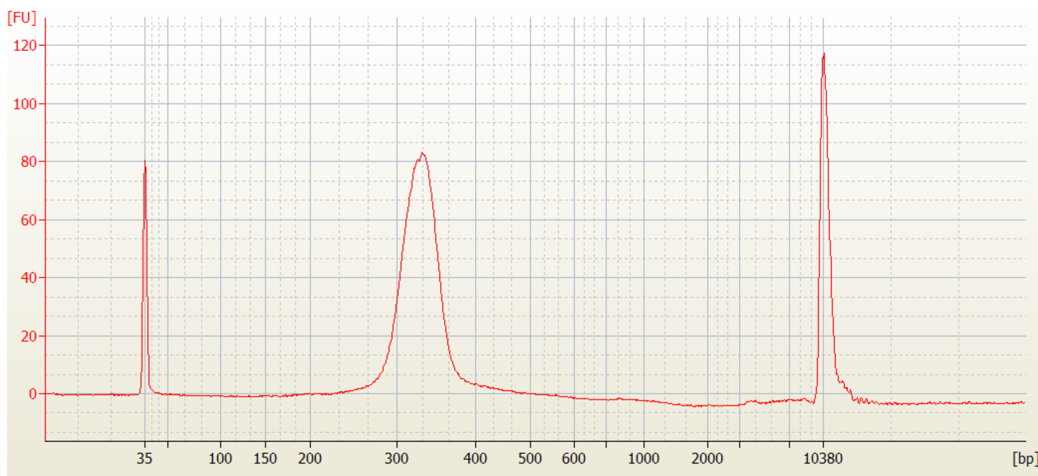


Figure 9 Bioanalyzer™ instrument analysis of a 200 base-read library— Bioruptor® System fragmentation and Pippin Prep™ size-selection

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

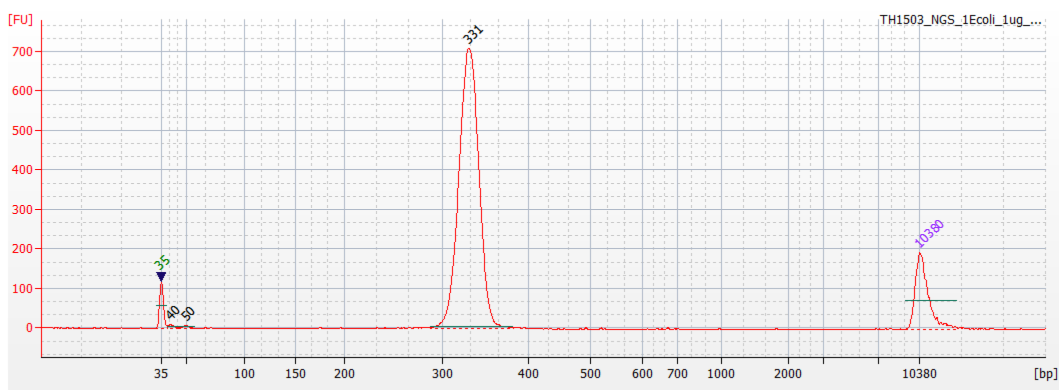
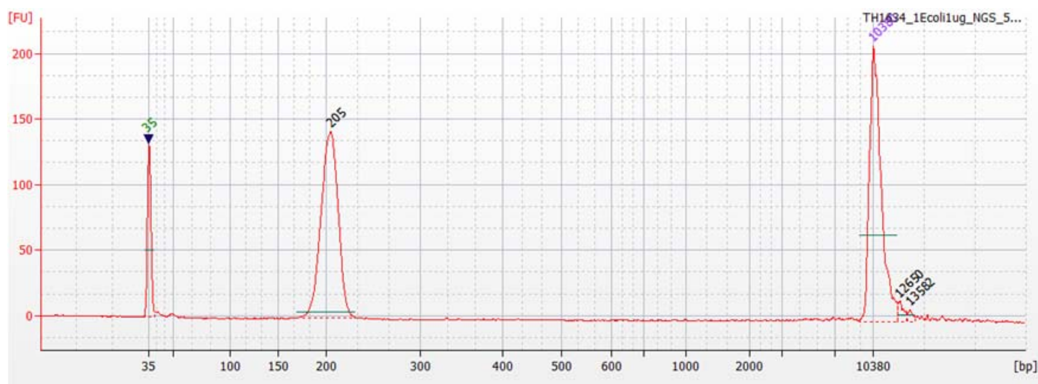


Figure 10 Bioanalyzer™ instrument analysis of a 100 base-read library— Bioruptor® System fragmentation and Pippin Prep™ size-selection

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



Appendix C Prepare short or long amplicon DNA

Amplicon design guidelines

- Use standard guidelines to design PCR primers for your region of interest. For design assistance, use a web tool such as Primer3:

  http://frodo.wi.mit.edu/primer3

- Design your primers so that any sequence variants of interest are located **between** the primers, so that those variants are not masked by the template-specific part of the primer sequences.
- Design short amplicons to be shorter than the median insert size for the desired target read length of the library. If you would like to sequence through the entire insert, design the amplicon to be shorter than the target read length.

Target read length	Median insert size
200 bases (200 base-read library)	~260 bp
100 bases (100 base-read library)	~130 bp

- When designing primers for longer amplicons, keep in mind that the primer sequence will not yield valuable sequence information, and the coverage of the ends of amplicons, the first 50 bases, is on average one-half of the average coverage.
- After pooling, long amplicons (>400 bp) require fragmentation with Ion Shear™ Plus Reagents before ligation to Ion-compatible adapters. Refer to **Fragment gDNA or long amplicons with the Ion Shear™ Plus Reagents** starting on page 14.
- After pooling, short amplicons (up to 250 bp) require end-repair before ligation to Ion-compatible adapters. Size selection of the libraries is not required. Refer to **Pool, end-repair, and purify short amplicons** starting on page 24.

Barcoding strategy for amplicon libraries

A barcoded library typically represents one biological specimen. The number of barcoded libraries (the number of biological specimens) that can be accommodated in a single sequencing run depends on the chip throughput, the size of the target region(s) of interest, and the coverage required. For a given chip throughput and coverage depth, as the size of the target region to be sequenced decreases, the number of barcoded libraries that can be accommodated per sequencing run increases. This relationship is shown in the following table, which describes the size of the target region in terms of the number of amplicons with an average length of 500 bp.

Table 1 Example of Ion Chip capacity for barcoded libraries for 500 bp amplicons

	Ion 314™ Chip		Ion 316™ Chip	
Desired coverage	100X (Germline)	1000X (Somatic)	100X (Germline)	1000X (Somatic)
Number of barcoded libraries	Approximate number of amplicons for 500 bp average size amplicon			
1	100	10	1000	100
2	50	5	500	50
3	33	3	333	33
4	25	2	250	25
5	20	2	200	20
6	16	1	166	16
7	14	1	142	14
8	12	1	125	12
9	11	1	111	11
10	10	1	100	10
11	9	—	90	9
12	8	—	83	8
13	7	—	76	7
14	7	—	71	7
15	6	—	66	6
16	6	—	62	6

PCR amplify genomic DNA targets and purify the amplicons

This section describes conditions for singleplex PCR amplification of genomic DNA to generate short (≤250 bp) or long (400–1000 bp) amplicons.

The amplification conditions described here are an example only. Use the procedure described here or your standard laboratory procedure, with the following guidelines.

- Avoid overamplification, which can generate single stranded DNA that cannot be fragmented properly for library construction.
- If many (for example, 12 or more) individually amplified amplicons will be pooled together for downstream library construction, consider using fewer amplification cycles (for example 25–35 cycles rather than 40 cycles).
- We strongly recommend using a high fidelity DNA polymerase, to reduce amplification errors.
- For amplicons 1000 bp–8 kbp in length, determine PCR conditions empirically or use the SequalPrep™ Long PCR Kit with dNTPs (Life Technologies/Invitrogen Part no. A10498).
- To save time and reagents, you can perform multiplex PCR if the primers have been specifically designed for multiplex PCR.

IMPORTANT! Multiplexing may increase the probability of unwanted PCR products and of over- or under-represented amplicons in the pool.

For each PCR reaction, start with 20–50 ng of high-quality, RNA-free genomic DNA.

Required materials and equipment

- Forward and reverse PCR primers
- 0.2 mL PCR strip tubes or 96-well Eppendorf® plate
- Platinum® PCR SuperMix High Fidelity
- Nuclease-free water
- Agencourt® AMPure® XP Reagent
- SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator
- 70% ethanol, freshly prepared

1. Thaw PCR primers, Platinum® PCR SuperMix High Fidelity, and high-quality genomic DNA on ice.
2. For each amplicon, mix equal volumes of the appropriate 10 µM forward and 10 µM reverse primers for a 10 µM primer stock mix (5 µM each primer).
3. Add the following reagents to 0.2-mL strip tubes or to the wells in a 96-well Eppendorf® plate exactly in this order:

Component	Volume
Platinum® PCR SuperMix High Fidelity*	45 µL
20–50 ng genomic DNA	4 µL
10 µM primer stock mix**	1 µL
Total	**50 µL**

* 5 µL total volume of primer and template in a 50-µL reaction is optimum. According to the Platinum® PCR SuperMix High Fidelity manual, no decrease in product yield is observed if the total volume of primer and template varies between 1 µL and 15 µL with 45 µL of Platinum® PCR SuperMix High Fidelity..
** If larger volumes of primer stock mix are desired for pipetting, use 5 µL of a 2-µM primer stock mix. Adjust the volume of Platinum® PCR SuperMix High Fidelity accordingly to keep the reaction volume at 50 µL.

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

   Note: Amplification conditions may vary according to primer design and DNA input. Adjust the cycling conditions and number of cycles for your specific experiment to achieve optimal results.

Stage	Step	Temperature	Time
Holding	Activate the enzyme	95ºC	3 min
Cycling (40 cycles)	Denature	95ºC	30 sec
	Anneal	58ºC	30 sec
	Extend	68ºC	1 min/kb
Holding	—	4ºC	∞

5. Purify the amplicon DNA with the Agencourt® AMPure® XP Kit.

IMPORTANT! If the total amplicon size, including target and primer sequence, is <100 bp, use a different purification method, such as the Qiagen MinElute® PCR Purification Kit.

a. Resuspend the Agencourt® AMPure® XP Reagent, and allow the mixture to come to room temperature (~30 minutes).

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

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

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

d. Place each plate or tube on a magnet (such as the Agencourt® SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator) for 3 minutes or until the solution clears. Remove and discard the supernatant from each well or tube without disturbing the bead pellet.

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

f. Repeat step 5.e for a second wash.

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

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

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

i. Remove the plate or tubes from the magnet, and add 15 µL of Nuclease-free Water directly to each bead pellet to disperse the beads. Pipet the mixture up and down 5 times to mix thoroughly.

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

j. Place the plate or tubes on a magnet for at least 1 minute. After the solution clears, **transfer the supernatant** containing the eluted DNA to a new plate or tube without disturbing the pellet.

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

STOPPING POINT (*Optional*) Store the DNA at –20°C.

6. Proceed to pooling the amplicons and library preparation.
    - For short amplicons up to 250 bp in length proceed to **Prepare an equimolar pool of short amplicons** starting on page 24.
    - For long amplicons >400 bp, proceed to **Fragment gDNA or long amplicons with the Ion Shear™ Plus Reagents** starting on page 14.

Appendix D Barcode discrimination

Torrent Suite Software v1.5 is recommended for sequence data analysis. The software includes tools for analysis of barcoded libraries prepared with the Ion Xpress™ Barcode Adapters 1–16.

The Ion Xpress™ Barcode Adapters 1-16 were designed for clear separation in flowspace. Barcodes are correctly assigned with high confidence in reads with up to 2 flowspace errors in the barcode region. In the rare situation of reads with four or more errors 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.

Safety

Chemical safety

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

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- 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. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/%2029cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: www.cdc.gov

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety

Manual, third edition, found at:
www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

Documentation and support

Related documentation

Visit the Ion Community **http://ioncommunity.iontorrent.com** and **www.appliedbiosystems.com/iontorrent** for the most up-to-date documentation for the Ion Torrent Personal Genome Machine™ System.

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.appliedbiosystems.com/sds** and **www.invitrogen.com/sds**.

For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining support

For the latest services and support information for all locations, go to **www.iontorrent.com/support/**.

At the website, you can:

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

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