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

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

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Overview

This user bulletin describes how to prepare Ion Torrent™-compatible libraries from amplicons that require fragmentation using the Ion Xpress™ Plus Fragment Library Kit (Cat. No. 4471269).

IMPORTANT!

- The protocol does not cover preparation of Ion AmpliSeq™ libraries. To prepare Ion AmpliSeq™ libraries, see the appropriate Ion AmpliSeq™ Library Kit User Guide. For more information, visit **thermofisher.com**.
- For information on using the Ion Xpress™ Plus Fragment Library Kit to prepare fragment libraries from genomic DNA, see the *Ion Xpress™ Plus gDNA Fragment Library Preparation User Guide* (Pub. No. MAN0009847).
- To prepare libraries from amplicons that do not require fragmentation, see the *Prepare Amplicon Libraries without Fragmentation Using the Ion Plus Fragment Library Kit User Bulletin* (Pub. No. MAN0006846).

Use the protocol in this user bulletin to perform the following steps in the library preparation workflow:

1. Prepare and purify amplicons. Generate amplicons that are at least 5 times longer than the targeted library insert length for efficient fragmentation. For example, to achieve efficient fragmentation and good coverage uniformity for a 200-base-read library, prepare amplicons that are at least 1,000 bp. See “Prepare and purify amplicons“ on page 9.
2. Pool the amplicons, shear the amplicons using the Ion Shear™ Plus Reagents, then re-purify. See “Pool, fragment, and purify the amplicons“ on page 11.
3. Ligate amplicons to Ion Torrent™-compatible adapters, then nick-repair the amplicons to complete the linkage between the adapters and inserts. For barcoded libraries, substitute adapters from the Ion Xpress™ Barcode Adapters Kit. See “Ligate adapters, nick repair, and purify“ on page 16.
4. Purify, then size-select the adapter-ligated library to achieve the optimal library length for your sequencing system. See “Size-select the unamplified library“ on page 19.
    - “Size-select the library with the E-Gel™ SizeSelect™ II Agarose Gel“ on page 19
    - “Size-select the library with the Pippin Prep™ System“ on page 26
5. After size selection, determine if amplification is required (see “Determine if library amplification is required“ on page 31). If needed, proceed to amplify and purify the library (see “Amplify and purify the library“ on page 32).
6. Quantify the library and dilute for template preparation.
    - For non-barcoded libraries, proceed to “Qualify non-barcoded libraries“ on page 34.
    - For barcoded libraries, proceed to “Qualify and pool barcoded libraries“ on page 36.

For more information, see “Workflow overview“ on page 7.

Template kit compatibility

The kits covered in the guide are compatible with all current Ion Torrent™-branded template preparation kits for the Ion Proton™ System, Ion PGM™ System, Ion S5™ System, Ion S5™ XL System, and Ion GeneStudio™ S5 Series System.

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

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

Kits used in this protocol

Ion Xpress™ Plus Fragment Library Kit

Ion Xpress™ Plus Fragment Library Kit (Cat. No. 4471269) provides reagents for preparing up to 20 libraries at 100-ng input, or up to 10 libraries at 1-µg input. Ion Xpress™ Plus Fragment Library Kit includes the components from the Ion Shear™ Plus Reagents Kit and the Ion Plus Fragment Library Kit:

Contents	Cap color	Amount	Storage
Ion Shear™ Plus Reagents Kit			
Ion Shear™ Plus 10X Reaction Buffer	Clear	2 × 50 µL	–30°C to –10°C
Ion Shear™ Plus Enzyme Mix II [1]	Clear	2 × 100 µL	
Ion Shear™ Plus Stop Buffer	Clear	2 × 50 µL	
Ion Plus Fragment Library Kit			
5X End Repair Buffer[2]	Red	400 µL	–30°C to –10°C
End Repair Enzyme[2]	Orange	20 µL	
10X Ligase Buffer	Yellow	200 µL	
DNA Ligase	Blue	40 µL	
Nick Repair Polymerase	Clear	160 µL	
dNTP Mix	Violet	40 µL	
Adapters	Green	100 µL	
Platinum™ PCR SuperMix High Fidelity	Black	2 × 1000 µL	
Library Amplification Primer Mix	White	100 µL	
Low TE	Clear	2 × 1.5 mL	15°C to 30°C *or* –30°C to –10°C

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

[2] Required only for physically fragmented gDNA.

(Optional) Ion Xpress™ Barcode Adapters Kits

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

The following Ion Xpress™ Barcode Adapters Kits are available:

- Ion Xpress™ Barcode Adapters 1–16 Kit (Cat. No. 4471250)
- Ion Xpress™ Barcode Adapters 17–32 Kit (Cat. No. 4474009)
- Ion Xpress™ Barcode Adapters 33–48 Kit (Cat. No. 4474518)
- Ion Xpress™ Barcode Adapters 49–64 Kit (Cat. No. 4474519)
- Ion Xpress™ Barcode Adapters 65–80 Kit (Cat. No. 4474520)
- Ion Xpress™ Barcode Adapters 81–96 Kit (Cat. No. 4474521)
- *(Complete set)* Ion Xpress™ Barcode Adapters 1–96 Kit (Cat. No. 4474517)

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

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

[1] X = barcode number

(Optional) Ion Plus Fragment Library Adapters

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

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

Required materials not supplied

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

Item	Source
Instruments and Equipment:	
Agilent™ 2100 Bioanalyzer™ instrument[1]	G2939BA
DynaMag™-2 Magnet (magnetic rack) *or* DynaMag™–96 Side Magnet	12321D 12331D
Microcentrifuge	MLS
Thermal cycler	MLS
Vortex mixer	MLS
Pipettors 1–1000 µL	MLS
Reagents and consumables:	
Agencourt™ AMPure™ XP Kit[2]	NC9959336 or NC9933872 fisherscientific.com
Agilent™ High Sensitivity DNA Kit	Agilent 5067-4626
Nuclease-free Water (not DEPC-Treated)	AM9932
(Optional) Ion Library TaqMan® Quantitation Kit[3] (required for quantification of unamplified libraries)	4468802
(Optional) 10 mM Tris, pH 7.5–8.5	MLS
Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5-mL	Fisher Scientific™ 13-698-791 Eppendorf 022431021
PCR tubes, 0.2-mL	MLS
Barrier pipette tips	MLS
***(Optional)* One of the following library preparation and templating controls:**	
Ion S5™ Controls Kit Plus	A30729
Ion PGM™ Controls Kit v3	A30046
Ion PI™ Controls 200 Kit	4488985
E. coli DH10B Control 600 Library	A32470

[1] Use to analyze DNA fragment length distribution during library preparation.

[2] Use for DNA purification.

[3] Not compatible with 600-base-read libraries.

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

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

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

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

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

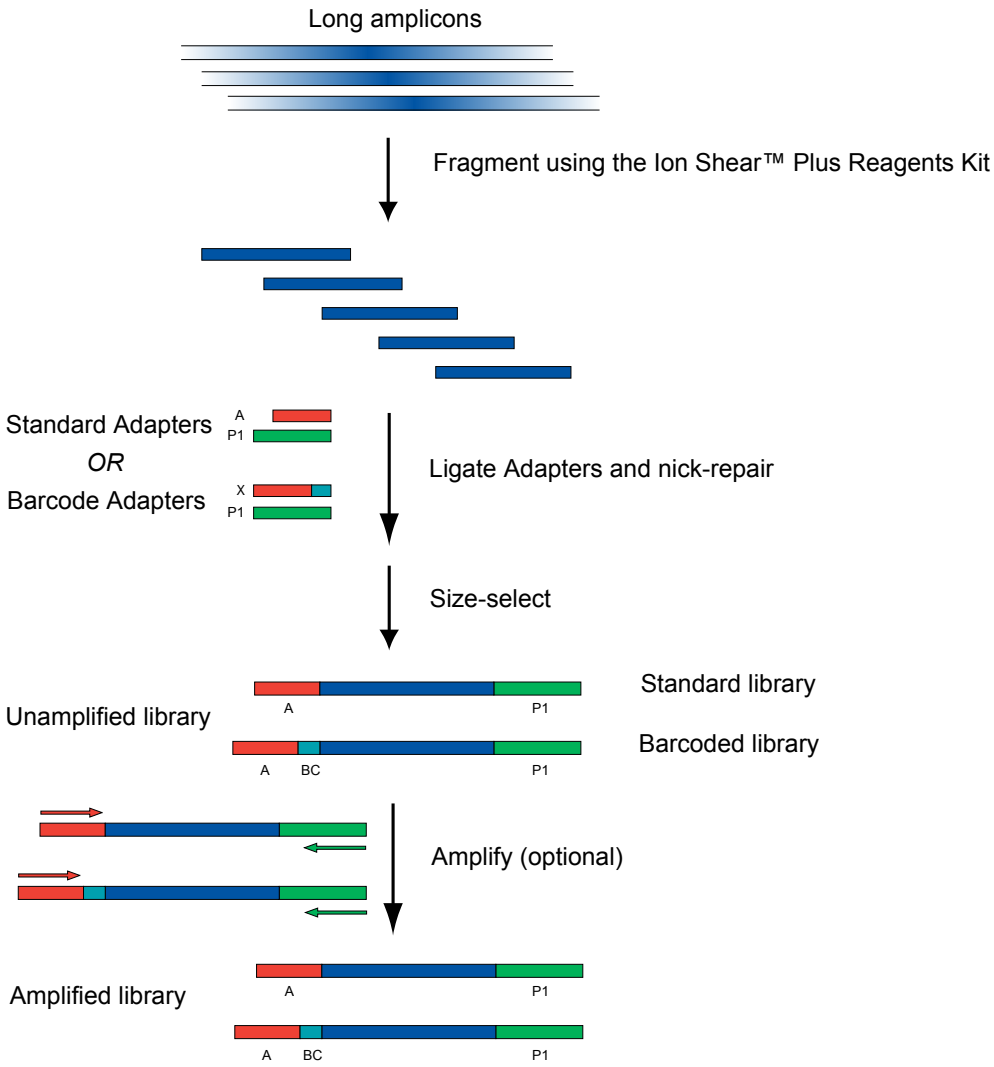
Required for size-selection using the Pippin Prep™ System

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

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

Workflow overview

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



Procedural guidelines

Guidelines – general

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

Guidelines for PCR

- Start with 20–50 ng of high-quality, RNA-free genomic DNA.
- Avoid overamplification, which can generate single-stranded DNA that cannot be fragmented properly for library construction.
- If 12 or more individually amplified amplicons will be pooled together for downstream library construction, consider using fewer amplification cycles (for example, 25–35 cycles instead of 40 cycles).
- We strongly recommend using a high-fidelity DNA polymerase to reduce amplification errors.
- For amplicons 1–8 kbp in length, determine PCR conditions empirically or use the SequalPrep™ Long PCR Kit with dNTPs (Cat. No. A10498).

Guidelines for PCR primer design

- Use standard guidelines to design PCR primers for your region of interest. For design help, use a web tool such as Primer3, available at **http://primer3.ut.ee/**
- Design your primers so that any sequence variants of interest are located between the primers, so that those variants are not masked by the template-specific part of the primer sequences.
- When designing primers for amplicons, keep in mind that the primer sequence will not yield valuable sequence information, and the coverage of the ends of amplicons—i.e., the first 50 bases—is on average one-half of the average coverage.
- We recommend that amplicon length is at least 5 times longer than the targeted library insert length for efficient fragmentation. For example, for a 200-base-read library, amplicon size should be at least 1000 bp for efficient fragmentation and good coverage uniformity.

Guidelines for purification

Following amplification, purify the amplicons.

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

Guidelines for barcoding amplicon libraries

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

Prepare and purify amplicons

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

Materials required

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

Example PCR protocol

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

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

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

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

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

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

Example amplicon purification protocol

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

Before you begin:

- Resuspend the Agencourt™ AMPure™ XP Reagent, then allow the mixture to come to room temperature (~30 minutes).
- Prepare 70% ethanol: 70 µL per amplicon (includes 10 µL of overage per amplicon).

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

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

2. Place each plate or tube on a magnet (such as the DynaMag™-2 Magnet or DynaMag™–96 Side Magnet) for 3 minutes or until the solution clears. Remove, then discard the supernatant from each well or tube without disturbing the bead pellet.

3. Without removing the samples from the magnet, dispense 150 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, then discard the supernatant without disturbing the pellet.

4. Repeat step 3 for a second wash.

5. 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.

6. 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.

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

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

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

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

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

Pool, fragment, and purify the amplicons

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

Materials required

The following materials are required for pooling:

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

The following materials are required for fragmentation and purification (provided in the Ion Xpress™ Plus Fragment Library Kit):

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

Other materials and equipment:

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

Prepare an equimolar pool of amplicons

Pooling amplicons in equimolar amounts for 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.

1. Using your laboratory practices or as described in the "Prepare and purify amplicons" on page 9, amplify gDNA targets of interest from 20–50 ng gDNA, then purify the individual amplicons. Use Nuclease-free Water for the final amplicon elution or resuspension.

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

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

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

3. Determine the concentration of the pooled amplicon stock.
   - Calculate the combined concentration of the pooled amplicons, then convert the concentration of the pooled amplicon stock to ng/µL.
   - Analyze 1 µL of the pooled DNA with the Agilent™ 2100 Bioanalyzer™ instrument and an Agilent™ High Sensitivity DNA Kit, then use the Bioanalyzer™ software to determine the molar concentration of the amplicon pool. If needed, use manual integration to place the entire range of amplicons within a single peak. Follow the manufacturer's instructions.

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

Guidelines for using the Ion Shear™ Plus Reagents

- Select the fragmentation conditions appropriate for the desired library size. The fragmented DNA is ready for adapter ligation.

Target Read Length	Median Insert Size
Ion S5™ System, Ion S5™ XL System, and Ion GeneStudio™ S5 Series System	
600 bases (600-base-read library)	550–650 bp
Ion PGM™ System, Ion S5™ System, Ion S5™ XL System, and Ion GeneStudio™ S5 Series System	
500 bases (500-base-read library)	470–570 bp
400 bases (400-base-read library)	~410 bp
300 bases (300-base-read library)	~320 bp
200 bases (200-base-read library)	~260 bp
100 bases (100-base-read library)	~130 bp
Ion Proton™ System	
200 bases (200-base-read library)	~200 bp
150 bases (150-base-read library)	~150 bp

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

Fragment the DNA

1. From the mass concentration that was calculated or determined during the pooling procedure, determine the volume for 100 ng of pooled amplicons.

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

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

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

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

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

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

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

   IMPORTANT! Do not mix by vortexing to avoid creating bubbles.

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

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

Median fragment size	Reaction time	Optimization range
550–650 (600-base-read library)	6 minutes	4–10 minutes
470–570 bp (500-base-read library)	7 minutes	4–12 minutes
350–450 bp (400-base-read library)	8 minutes	5–12 minutes
270–370 bp (300-base-read library)	10 minutes	5–15 minutes
200–300 bp (200-base-read library)	15 minutes	5–30 minutes
150–250 bp (150-base-read library)	20 minutes	10–40 minutes
100–200 bp (100-base-read library)	40 minutes	30–60 minutes

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

Purify the fragmented DNA

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

Prepare 1 mL plus overage of 70% ethanol per sample.

1. Add 99 µL of Agencourt™ AMPure™ XP Reagent (1.8X sample volume) to the sheared DNA sample, then pipet up and down five times to thoroughly mix the bead suspension with the DNA. Pulse-spin to collect the contents at the bottom of the tube, then incubate the mixture at room temperature for 5 minutes.
2. Pulse-spin, then place the tube in a magnetic rack, such as the DynaMag™-2 Magnet, for 3 minutes or until the solution is clear of brown tint when viewed at an angle. Carefully remove, then discard the supernatant without disturbing the bead pellet.
3. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol.
4. Incubate for 30 seconds, turning the tube twice in the magnet to move around the beads. After the solution clears, remove, then discard the supernatant without disturbing the pellet.
5. Repeat step 3–step 4 for a second wash.
6. To remove residual ethanol, pulse-spin the tube, place the tube back in the magnetic rack, then carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
7. Keeping the tube on the magnet, air-dry the beads at room temperature for 3–5 minutes.
8. Remove the tube from the magnetic rack, then add 25 µL of Low TE directly to the pellet to disperse the beads. Pipet the suspension up and down five times, then vortex the sample for 10 seconds to mix.
9. Pulse-spin, then place the tube in the magnetic rack for at least 1 minute or until the solution clears. Transfer the supernatant containing the eluted DNA to a new 0.2-mL PCR tube without disturbing the pellet.

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

10. *(Optional)* Check the fragment size using 1 µL of eluted DNA with the Agilent™ 2100 Bioanalyzer™ instrument and the Agilent™ High Sensitivity DNA Kit. Confirm that your DNA fragment size is within the appropriate range:

Sequencing system	Library type	Target median fragment size	Fragment size range
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System	600-base-read library	550–650 bp	200–1,200 bp
Ion PGM™ System Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System	500-base-read library	470–570 bp	150–1,000 bp
	400-base-read library	350–450 bp	150–1,000 bp
	300-base-read library	270–370 bp	100–900 bp
	200-base-read library	200–300 bp	100–700 bp
	100-base-read library	100–200 bp	50–500 bp
Ion Proton™ System	200-base-read library	150–250 bp	100–700 bp
	150-base-read library	100–200 bp	50–500 bp

Proceed to "Ligate adapters, nick repair, and purify".

Ligate adapters, nick repair, and purify

Materials required

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

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

The following materials are included in the Ion Xpress™ Barcode Adapters Kits *(for barcoded libraries only)*

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

Other materials and equipment:

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

Ligate and nick repair

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

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

[1] X = barcode chosen.

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

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

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

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

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

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

Purify the adapter-ligated and nick-repaired DNA

IMPORTANT! Use freshly prepared 70% ethanol.

Prepare 1 mL plus overage of 70% ethanol per sample.

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

Library size	Volume of Agencourt™ AMPure™ XP Reagent
400–600-base-read	100 µL (1X sample volume)
200–300-base-read	120 µL (1.2X sample volume)
100–150-base-read	150 µL (1.5X sample volume)

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

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

4. Repeat step 3 for a second wash.

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

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

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

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

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

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

Proceed to "Size-select the unamplified library".

Size-select the unamplified library

Use one of the following methods to size-select the unamplified barcoded or non-barcoded library:

- "Size-select the library with the E-Gel™ SizeSelect™ II Agarose Gel" on page 19
- "Size-select the library with the Pippin Prep™ System" on page 26

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

Sequencing system	Library size	Target peak size
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System	600-base-read	~680 bp
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System Ion PGM™ System	500-base-read	~580 bp
	400-base-read	~480 bp
	300-base-read	~390 bp
	200-base-read	~330 bp
	100-base-read	~200 bp
Ion Proton™ System	200-base-read	~270 bp
	150-base-read	~220 bp

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

Materials required

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

Procedural guidelines

- Use the indicated amount of DNA per well for single or multiple bands:
  - Do not exceed 500 ng of total DNA per one sample lane.
  - For sheared DNA, do not exceed 1 µg.
- For 500-base-read libraries, follow the recommendations for 600-base-read libraries.

- Samples that contain ≥50-mM NaCl, 100-mM KCl, 10-mM acetate ions, or 10 mM EDTA (for example, certain restriction enzymes and PCR buffers) can cause loss of resolution on E-Gel™ agarose gels. For best results, dilute samples that contain high salt levels 2- to 5-fold in low TE.
- Load the E-Gel™ SizeSelect™ II Agarose Gel within 15 minutes of opening the pouch. Run the gel within 1 minute of loading the samples.
- Always wear Safe Imager™ Viewing Glasses when working with the E-Gel™ Power Snap Electrophoresis Device with the cover open.

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

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

Prepare samples

Prepare up to 25 µL of sample in 1X Sample Loading Buffer.

IMPORTANT! This step is required, do not omit.

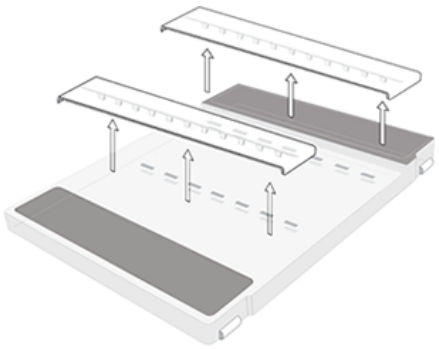
Component	Volume
10X Sample Loading Buffer	2.5 µL
Sample (from step 8 on page 18)	20 µL
UltraPure™ DNase/RNase-Free Distilled Water	up to 25 µL

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

Prepare the gel

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

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



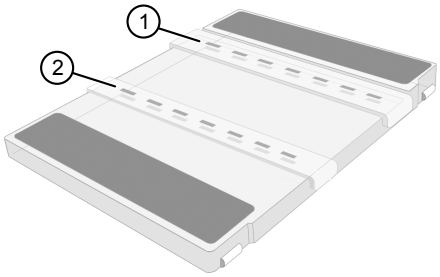
3. Insert the gel cassette into the E-Gel™ Power Snap Electrophoresis Device, starting from the right edge.

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

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

Load samples

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

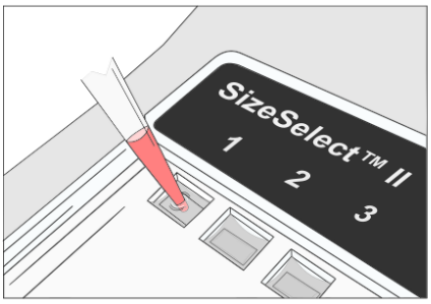


① Sample wells
② Recovery wells

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

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

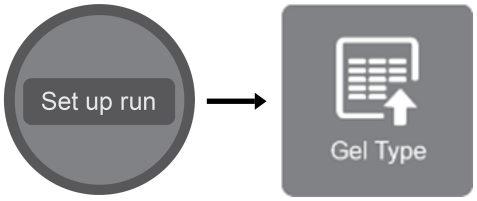
IMPORTANT! Do not pierce the gel or introduce bubbles into the wells.



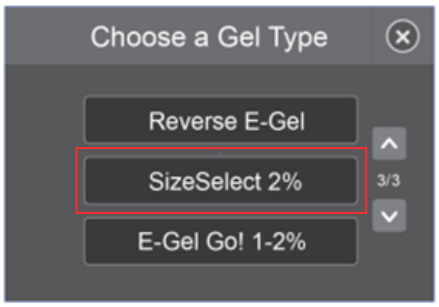
3. Load 25 µL of ready-to-use E-Gel™ Sizing DNA Ladder into the marker well.

Run the gel

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



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

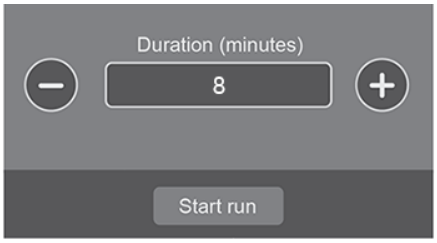


3. Determine the estimated DNA run time. For more information, see "Guidelines for estimating run time" on page 24 .

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

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

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



Check the gel status

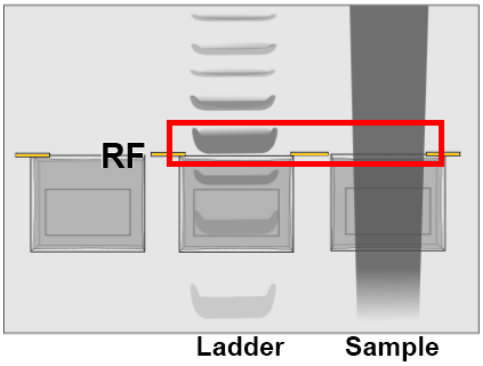
You can monitor the gel during the run to avoid the target fragment passing the recovery well.

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

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

   Note: The transilluminator turns off automatically after 1 minute.

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



Prepare the recovery wells

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

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

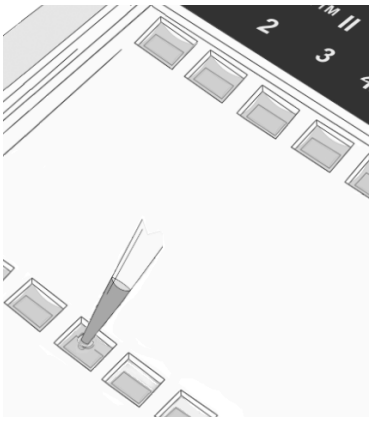
   Note: The transilluminator turns off automatically when the filter lid is opened. Tap **Back light** to re-activate the blue light transilluminator.

2. Carefully remove all liquid from each recovery well.

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

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

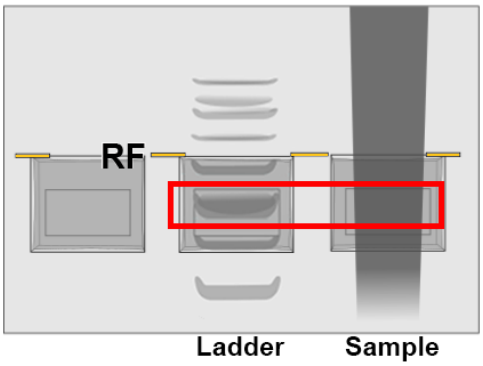
   IMPORTANT! Do not allow water to spill over the edge of the wells.



Collect the DNA fragment

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

   IMPORTANT! To determine when to collect samples of specific target library length, see Table 3 in "Guidelines for estimating run time" on page 24.



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

   IMPORTANT! Avoid piercing the agarose during collection.

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

Proceed to "Determine if library amplification is required" on page 31.

Guidelines for estimating run time

- To estimate target DNA run time to the reference line, see Table 2.
- The E-Gel™ Sizing DNA Ladder is also used as a size reference marker. To estimate run time from the reference line to the collection well for 50–100-ng sample input, see Table 3.
- The run times that are indicated in the tables are estimates. Monitor your gel in real-time during the run to ensure that the sample does not pass the recovery well.
- Identically sized bands in different wells can migrate differently.
- DNA fragment size, amount, and salt content can affect migration rates.

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

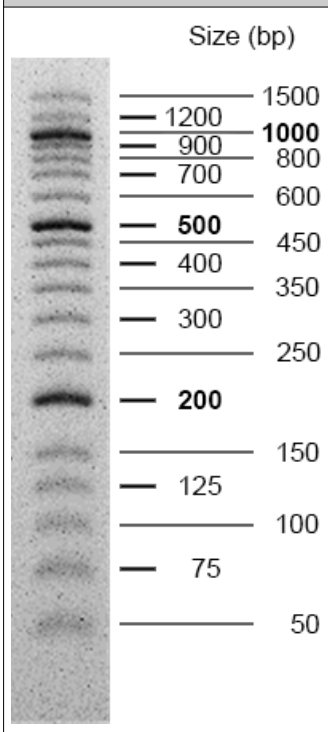
Ladder	Fragment size	DNA amount (per 25 µL)	Estimated migration time to reference line
	1,500 bp	1.5 ng	~ 19.5 minutes
	1,200 bp	1.5 ng	~ 18.5 minutes
	1,000 bp	6 ng	~ 17.5 minutes
	900 bp	2 ng	~ 17 minutes
	800 bp	2 ng	~ 16.5 minutes
	700 bp	2 ng	~ 16 minutes
	600 bp	2 ng	~ 15.5 minutes
	500 bp	6 ng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

Table 3 NGS library size selection reference

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

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

Size-select the library with the Pippin Prep™ System

Materials required

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

Set up the instrument and perform the run

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

IMPORTANT!
- This procedure closely follows the Pippin Prep™ System manual.
- Software version 3.71 or later is required to turn off signal monitoring of the sample lanes.

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

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

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

Sequencing system	Library size	BP Target setting
Ion S5™ System Ion S5™ XL System Ion GeneStudio™ S5 Series System Ion PGM™ System	300-base-read	390 bp
	200-base-read	315 bp
	100-base-read	180 bp
Ion Proton™ System	200-base-read	270 bp
	150-base-read	220 bp

     c. Define lanes 1–4 as sample lanes and 5 as the ladder lane by entering ***5*** in the reference lane field, then selecting the **Apply Reference to all Lanes** button. Ensure that the **Ref Lane** value for each lane is **5**.
     d. Set the run time to 1.5 hours.
   - For 400- and 500-base-read libraries:
     a. From the cassette type dropdown list, select **2% DF Marker L**.
     b. Select the **Tight** collection mode for each lane, then define the **BP Target** setting for each of 1–5 lanes used.

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

c. Define lanes 1–5 as sample lanes, then press the **Use Internal Standards** button to match the lane numbers, then ensure that the **Ref Lane** values match the lane numbers.

d. Set the run time to 1.5 hours.

- For 600-base-read libraries:
  a. From the cassette type dropdown list, select **1.5% DF Marker K**.
  b. Select the **Tight** collection mode for each lane, then define the **BP Target** setting for each lane as **685 bp**.

     Note: The Pippin Prep™ instrument can under- or over-select this target. Adjust your **BP Target** setting based on your laboratory conditions.

  c. Define lanes 1–5 as sample lanes, press the **Use Internal Standards** button to match the lane numbers, then ensure that the **Ref Lane** values match the lane numbers.

  d. Set the run time to 1.5 hours.

2. Prepare the Agarose Gel cassette.

   Note: For 600-base-read libraries, use the 1.5% Agarose Gel cassettes. For 500-base-read libraries or smaller, use the 2% Agarose Gel cassettes.

   a. Unwrap the 1.5% or 2% Agarose Gel cassettes, then tip the cassette toward the loading wells end to dislodge any air bubbles present near the elution wells. Tap the bottom of the cassette until there are no bubbles that remain behind the elution wells.

   b. Insert the cassette into the instrument.

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

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

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

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

   g. Following the instructions in the Pippin Prep™ System user guide, make sure that the current across both the separation ports and the elution ports is within specifications.

3. Load the sample.

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

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

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

- For 400- and 500-base-read libraries:
  a. Add 10 µL of Low TE to the purified ligated DNA (20 µL) to bring the volume to 30 µL.
  b. Add 10 µL of Loading Solution/marker mix (labeled Marker L). The total volume is 40 µL for each sample.
  c. Go to the **Main** screen, then select the newly generated separation file (or a previously saved file) from the **Protocol Name** dropdown list.
  d. Remove 40 µL of Electrophoresis Buffer from one sample loading well at a time, then immediately load the entire 40-µL sample into the well.

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

- For 600-base-read libraries:
  a. Add 10 µL of Low TE to the purified ligated DNA (20 µL) to bring the volume to 30 µL.
  b. Add 10 µL of Marker K. The total volume is 40 µL for each sample.
  c. Go to the **Main** screen, then select the newly generated separation file (or a previously saved file) from the **Protocol Name** dropdown list.
  d. Remove 40 µL of Electrophoresis Buffer from one sample loading well at a time, then immediately load the entire 40-µL sample into the well.

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

4. Run the instrument.
   a. When the ladder and all samples are loaded, close the lid of the Pippin Prep™ instrument.
   b. On the **Main** screen, press **Start** to begin the run.
   c. When the separation is complete, transfer the DNA from each elution well (typically 40–60 µL) with a pipettor to new 1.5-mL Eppendorf LoBind™ tube.
   d. Add Nuclease-free Water to the DNA to bring the volume to 60 µL.

Purify the size-selected DNA *(for Pippin Prep™ size-selection only)*

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

1. Add 108 µL of Agencourt™ AMPure™ XP beads (1.8X sample volume) to each sample, then pipet up and down five times to mix the bead suspension thoroughly with the DNA. Pulse-spin the tube, then incubate the mixture for 5 minutes at room temperature.
2. Pulse-spin, then place the tube in a magnetic rack such as the DynaMag™-2 Magnet for 3 minutes or until the solution is clear. Carefully remove, then discard the supernatant without disturbing the pellet.
3. Without removing the tube from the magnet, add 500 µL of freshly prepared 70% ethanol to the sample.
4. Incubate for 30 seconds, turning the tube twice in the magnet to move the beads around. After the solution clears, remove, then discard the supernatant without disturbing the pellet.
5. Repeat step 3–step 4 for a second wash.
6. To remove residual ethanol, pulse-spin the tube, place it back in the magnetic rack, then carefully remove any remaining supernatant with a 20-µL pipettor without disturbing the pellet.
7. Keeping the tube on the magnet, air-dry the beads at room temperature for ≤5 minutes.
8. Remove the tube from the magnetic rack, then add 25 µL of Low TE directly to the pellet to disperse the beads. Pipet the mixture up and down five times, then vortex the sample for 10 seconds to mix thoroughly.
9. Pulse-spin, then place the tube in the magnetic rack for at least 1 minute until the solution clears. Transfer the supernatant containing the eluted DNA to a new 1.5-mL Eppendorf LoBind™ tube without disturbing the pellet.

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

Proceed to "Determine if library amplification is required".

Determine if library amplification is required

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

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

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

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

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

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

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

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

Library amplification	Proceed to...
Yes	"Amplify and purify the library" on page 32
No	• "Qualify non-barcoded libraries" on page 34 *or* • "Qualify and pool barcoded libraries" on page 36

Amplify and purify the library

Materials required

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

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

Other materials:

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

Amplify the library

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

Component	Volume
Platinum™ PCR SuperMix High Fidelity	100 µL
Library Amplification Primer Mix	5 µL
Unamplified library (size-selected with the E-Gel™ SizeSelect™ II Agarose Gel or Pippin Prep™ System)	25 µL
Total	**130 µL**

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

3. Place the tubes into a thermal cycler, then run the following PCR cycling program.

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

Stage	Step	Temperature	Time
Hold	Denature	95°C	5 min
Cycle (8 cycles)	Denature	95°C	15 sec
	Anneal	58°C	15 sec
	Extend	70°C	1 min
Hold	—	4°C	Hold for up to 1 h

4. Combine the previously split PCR reactions in a new 1.5-mL Eppendorf LoBind™ tube.

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

Purify the library

1. Add 195 µL of Agencourt™ AMPure™ XP Reagent (1.5X sample volume) to each sample, pipet up and down five times to thoroughly mix the bead suspension with the DNA. Pulse-spin, then incubate the mixture for 5 minutes at room temperature.

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

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

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

5. Repeat step 3–step 4 for a second wash.

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

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

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

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

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

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

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

- For non-barcoded libraries, proceed to "Qualify non-barcoded libraries" on page 34.
- For barcoded libraries, proceed to "Qualify and pool barcoded libraries" on page 36.

Qualify non-barcoded libraries

Evaluate the quality of the library

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

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

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

Determine the library dilution required for template preparation

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

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

  IMPORTANT! The Ion Library TaqMan® Quantitation Kit is not compatible with 600-base-read libraries.

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

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

If you perform both procedures:

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

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

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

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

   Determine the dilution factor using the following formula:

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

   Example:

   The library concentration is 15,000 pM.

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

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

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

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

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

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

   Determine the dilution factor using the following formula:

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

   Example:

   The library concentration is 15,000 pM.

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

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

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

Proceed to template preparation

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

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

The libraries are ready for downstream template preparation using an appropriate Ion Torrent™-branded template preparation kit.

Qualify and pool barcoded libraries

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

IMPORTANT!

- Unamplified libraries must be quantified for pooling with the Ion Library TaqMan® Quantitation Kit (Cat. No. 4468802).
- The Ion Library TaqMan® Quantitation Kit is not compatible with 600-base-read libraries.

For non-barcoded libraries, go to "Qualify non-barcoded libraries" on page 34.

Assess the quality of individual barcoded libraries

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

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

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

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

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

1. Use the Ion Library TaqMan® Quantitation Kit (Cat. No. 4468802) to determine library concentration in pmol/L by quantitative real-time PCR (qPCR) for each individual barcoded library. Follow the instructions in the *Ion Library TaqMan® Quantitation Kit User Guide* (Pub. No. MAN0015802).
2. Dilute each barcoded library to a concentration of ~100 pM. This concentration is appropriate for downstream template preparation.

   Determine the dilution factor using the following formula:

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

   Example:

   The barcoded library concentration is 15,000 pM.

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

   Therefore, 1 µL of barcoded library that is diluted with 149 µL of Low TE (1:150 dilution) yields a 100-pM solution.
3. Prepare at least 20 µL of a barcoded library pool by mixing equal volumes of the diluted barcoded libraries.

   The library pool is at the correct concentration for template preparation using Ion Torrent™-branded template preparation kits.

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

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

   Note: If needed, follow the manufacturer's instructions to perform a region analysis (smear analysis) to place the entire distribution of library molecules within a single peak.
2. Prepare an equimolar pool of barcoded libraries at the highest possible concentration.

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

3. Determine the molar concentration of the library pool.
   - Use the combined concentration of the library pool that was calculated for your library pooling algorithm.
   - Alternatively, confirm the concentration of the library pool by analyzing 1 µL of the library pool on the Agilent™ 2100 Bioanalyzer™ instrument with an Agilent™ High Sensitivity DNA Kit.

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

4. Dilute the library pool to a concentration of ~100 pM.

   Determine the dilution factor using the following formula:

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

   Example:

   The library pool concentration is 15,000 pM.

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

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

   This concentration is appropriate for downstream template preparation.

Proceed to template preparation

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

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

The libraries are ready for downstream template preparation using an appropriate Ion Torrent™-branded template preparation kit.

Supplemental information

Barcode discrimination

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

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

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

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

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Revision history: Pub. No. MAN0007044

Revision	Date	Description of Change
C.0	11 April 2019	• Updated the list of required materials and ordering information in "Required materials not supplied". • Updated the "Overview" topic to include protocol references. • Replaced the discontinued E-Gel™ SizeSelect™ 2% Agarose Gel (Cat. No. G661002) with E-Gel™ SizeSelect™ II 2% Agarose Gel (Cat. No. G661012). • Replaced the discontinued E-Gel™ iBase™ and E-Gel™ Safe Imager™ Combo Kit with E-Gel™ Power Snap Electrophoresis Device. • Updated the protocol in "Size-select the library with the E-Gel™ SizeSelect™ II Agarose Gel" to accommodate the E-Gel™ SizeSelect™ II 2% Agarose Gel and the E-Gel™ Power Snap Electrophoresis Device. • Changed ordering information for the Pippin Prep™ System and associated kits. • Updated sequencer information to include Ion GeneStudio™ S5 Series System. • Added recommendations for 500- and 600-base-read libraries throughout. • Removed the *Ion non-barcoded and barcode adapter sequences* supplemental information.
B.0	28 April 2016	• Added support for Ion S5™ and Ion S5™ XL Systems • General rebranding and streamlining
A.0	3 March 2014	• Added support for 400-base-read libraries. • Added support for the Ion Proton™ System. • Version numbering changed to alphanumeric format and reset to A.0 in conformance with internal document control procedures.

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